DOWNREGULATION OF *REPRODUCTIVE HOMEOBOX GENE 6 (RHOX6)* INTERFERES WITH MALE GERM CELL DIFFERENTIATION

BY

CHANG LIU

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Animal Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Master's Committee:
Assistant Professor Tetsuya S. Tanaka, Chair
Professor Janice M. Bahr
Associate Professor David J. Miller

ABSTRACT

Homeodomain proteins contain a 60 amino acid DNA binding motif called homeobox that regulates a variety of developmental events. Recently, a novel cluster of homeobox genes has been discovered on the X chromosome of the mouse. Because of their selective expression in reproductive organs, they are named *Reproductive homeobox genes on the X chromosome (Rhox*). *Rhox6*, a member of the *Rhox* family, is expressed in primordial germ cells (PGCs) in the developing gonad and the placenta, although the biological function of *Rhox6* in determination of PGCs and placental cells remains unknown. To investigate the *Rhox6* function, we generated male chimeras by injection of the gene-trapped embryonic stem cells (ESCs) harboring a hypomorphic mutation in *Rhox6* into wild-type blastocysts. Almost all of the male chimeras exhibited dramatically reduced fertility associated with smaller testes, reduced sperm number and motility and abnormal morphology of the epididymal sperm. To obtain a better understanding of the *Rhox6* function in germ cell differentiation, we took advantage of *in vitro* ESC culture. ESCs can differentiate into PGCs when ESCs are cultured as aggregates called embryoid bodies (EBs). By using a mouse ESC line that expresses the enhanced green fluorescent protein (EGFP) under the promoter of *Oct3/4*, a central regulator of cellular pluripotency, the presence of PGCs within EBs was monitored in real time by EGFP expression. While this ESC line was induced to differentiate into PGCs, expression of *Rhox6* was downregulated by stable expression of the short-hairpin (sh) RNA against the *Rhox6* transcripts and we found that continuous downregulation of *Rhox6* expression resulted in dramatic decrease in the number of germ cells differentiated. Collectively, these results suggest that downregulation of *Rhox6* impairs differentiation of PGCs.

ACKNOWLEDGEMENT

First and foremost I would like to thank my advisor, Dr. Tetsuya Tanaka, for his guidance and support over the past one year. I truly appreciate his instruction and inspiration throughout my studies and research. I would like to thank all the current and previous Tanaka Lab members for their help and support as labmates and friends. I thank Paige Tsai for her technical support in the research. I thank Ana Marie García Rivera, Brandon Logeman for their collaborative effort on the work presented in this thesis. I thank Dr. Miller for sharing reagents and protocol and his instruction in doing experiment.

My appreciation also goes to Illinois Regenerative Medicine Institute and Department of Animal Sciences for all the support and resources during the research. I thank the Travel Award and the Labor Foundation Fellow Award from the Society for the Study of Reproduction.

I would like to thank my wife, my family for their love, support and understanding. None of my accomplishments would have been possible without their support. I also want to thank Dr. Bahr for her encouragement and mentoring which helped me regain my strength to pursue my career in science.

TABLE OF CONTENTS

CHAPTER 1

Literature review

About 15% of couples are unable to conceive spontaneously (Cooke and Saunders, 2002), whereas up to two thirds of males among such couples suffer from infertility (Bhasin et al., 1994). Because genetic mutations such as chromosomal aberrations and single nucleotide substitutions have been identified in male patients suffering from infertility (Ferlin et al., 2007), a better understanding of male germ cell differentiation with respect to gene expression may lead to development of innovative and effective means to treat male infertility. To achieve this goal, I summarize our current knowledge in male germ cell differentiation and identify critical needs.

Mouse Embryonic Development and Origin of The Germ Cell Lineage

In many animal species, embryonic development begins with fertilization of an egg by a sperm. By 24 hours after fertilization, human and mouse embryos complete the first cell division cycle and will continue to divide once every 12 hours for mouse and 24 hours for human in average until they reach the uterus for implantation. It usually take three to four days for the mouse zygote to make its way to the uterus and, for human zygotes, five to seven days, when these embryo develop into blastocysts. Once blastocysts implant, they grow much faster due to proliferation and differentiation of pluripotent cells, known as the epiblast, from which the embryo proper will develop. This differentiation process is initiated by the critical event called gastrulation. Gastrulation begins seven days after fertilization in mouse embryos, and between days 14 and 16 of human development. Gastrulation establishes the basic body plan and organ primordium of the future body (Hogan et al., 1994; Gilbert, 2000).

Differentiation of germ cells is initiated during gastrulation. These germ cell precursors start to migrate toward the gonadal primordium, genital ridges, wherein they become known as gonocytes (McLaren, 2003). However, the precise events and timing of germ cell lineage establishment in human embryos remain to be investigated.

In mouse embryos, founder population of primordial germ cells (PGCs) are determined at the early stage of embryogenesis and set aside from the rest of the body part (Fujimoto et al, 1977; Aflatoonian and Moore, 2006; Ohinata et al. 2006; Saitou et al. 2009). The PGCs are specified at the posterior end of the extending primitive streak during gastrulation at 7.2 days after fertilization (Saitou et al., 2002). These cells then enter the epithelium of the future hind-gut, migrate along the wall of the developing gut while proliferating, localize into the genital ridges and eventually develop into functional gametes (Anderson et al., 2000). The determination, proliferation, migration and differentiation of PGCs determine the number and the function of mature gametes. Thus, elucidating the mechanism of PGC determination and differentiation is crucial for understanding the etiology of various aspects of infertility (Grimaldi et al., 2002; Ferlin et al., 2007).

Specification of Primordial Germ Cells

During differentiation events from establishment of the germ cell fate to production of haploid spermatozoa, germ cells start their migration to reach future gonads. Afterward, germ cells experience dramatic morphological changes, which result in reducing the diploid set of chromosomes into a haploid set. Importantly, during this event homologous chromosomes cross over and recombine. Thus, the biological diversity is retained by generating the haploid genome.

Cell type- and stage-specific transcriptional factors are involved in many of these events (Maclean and Wilkinson, 2005).

Specification of the germ-line by repression of the program to differentiate somatic cells

In the experimental model organisms such as fruit fly (*Drosophila melanogaster*), the soil roundworm (*Caenorhabditis elegans*), African clawed frog (*Xenopus laevis*), and zebrafish (*Danio rerio*), germ cells are specified by the specific cytoplasm localized in oocytes, named "germ plasm". It contains mitochondria and unique electron-dense granules consisting of uncharacterized RNA and proteins. These granules are variously referred to as germinal granules in *Xenopus*, polar granules in *Drosophila* and P-granules in *C. elegans* (Wylie, 2000). On the other hand, in other experimental model organisms such as newts and mice, specification of primordial germ cells is determined not by inheritance of the germ plasm, but by an inducing signal from neighboring cells, which is a popular mode for germ cell specification in metazoans (Extavour and Akam, 2003; Tam and Zhou, 1996).

Despite the differences in specification of PGCs among these experimental models, PGC specification may depend on the common mechanism, which is transcriptional repression of genes expressed in somatic cells (Seydoux and Braun, 2006). There are two independent modes of transcriptional repression in germ cells: one early mode involving direct inhibition of RNA polymerase II and a later mode regulates at the level of chromatin modification (Blackwell, 2004; Schaner and Kelly, 2006). In the first mode, for instance, the transcriptional block in *C. elegans* depends on *pie-1,* a germ plasm component inherited maternally. When transcription was turned on in PGC precursors in *pie-1* mutants, PGCs were not differentiated (Blackwell, 2004). The loss of PGCs in the *pie-1* mutant *C. elegans* embryos was due to transformation of germ cell

precursors into somatic cells (Schaner and Kelly, 2006). Similarly, in mice, PGCs do not express *Hox* gene transcripts, which are actively transcribed in neighboring somatic cells, suggesting that downregulation of *Hox* gene transcripts is essential for PGC specification (Ohinata et al., 2006). In the later mode, for example, in *Drosophila* and *C. elegans,* early germ cells have reduced level of H3-K4me2 (dimethylation of lysine 4 on histone H3), a methyl mark linked with transcription. In *C. elegans,* H3-K4me2 levels drop only in later stage after *pie-1* disappears from the germ lineage and PGCs exhibit an elevated ratio of trimethylated to dimethylated H3-K27, a mark correlated with transcriptional repression (Schaner and Kelly, 2006). Collectively, these experimental evidences suggest that global repression of the program to differentiate somatic cells is essential to promote the fate of germ cells.

Genetic regulation of PGC specification

Signaling molecules involved in primordial germ cell specification

In mouse embryos, cellular competency to differentiate into germ cells is retained in the proximal epiblast cells at 7 days after fertilization. An inducing signal that emanates from the extraembryonic ectoderm (McLaren, 1999; Yoshimizu et al., 2001) is necessary to direct the fate of the proximal epiblast cells into the germ cell lineage. However, these proximal epiblast cells at this stage are not lineage restricted, as they give rise to primordial germ cells (PGCs) as well as somatic cells (Lawson and Hage, 1994).

Gene knockout studies have demonstrated that a member of the transforming growth factor β (*TGFβ*) family, Bone morphogenetic protein 4 (*Bmp4*), and its downstream target transcription factor *Smad5*, are essential for PGC specification (Lawson et al., 1999; Chang et al., 2002; Zhao, 2003). *Bmp4* homozygous null mouse embryos develop no PGCs. Additionally, *Bmp4*-knockout

embryos failed to differentiate the extraembryonic mesodermal tissue, allantois, from precursors in the proximal epiblast. In *Bmp4* heterozygotes, there were fewer number of PGCs formed due to a reduced size of the founder population, whereas there was no effect detected on its subsequent expansion (Lawson et al., 1999). *Bmp4* is initially expressed in the extraembryonic ectoderm (ExE) directly contacting the proximal epiblast from around 5.5 days after fertilization (Lawson et al., 1999). Its expression is further detected in the extraembryonic mesoderm (ExM) of mid- to late- primitive streak stage embryos around 7 days after fertilization. However, no expression of *Bmp4* was detected in PGCs (Lawson et al., 1999; Fujiwara et al., 2001). Therefore these observations suggest that the initiation of germ cell specification in the mouse depends on a secreted signal from extraembryonic lineage (Lawson et al., 1999).

In addition to *Bmp4*, *Bmp8b*, which is expressed exclusively in the ExE as early as 5.5 days after fertilization and *Bmp2*, which is expressed primarily in the visceral endoderm (VE) of pregastrulating and gastrulating embryos, also play important roles in establishment of the germ cell lineage (Ying et al., 2000; Ying and Zhao, 2001). These findings indicate that at least three *Bmps, Bmp4, Bmp8b* and *Bmp2* are necessary for determination of PGCs *in vivo*. However, it is still not clear through which receptors these *Bmps* signal to specify PGCs. Thus of critical importance is to understand how initiation of PGC specification is regulated through transcription factors in the *Bmp* signaling pathway.

Accordingly, there are several *Smad* proteins transducing the Bmp signals. For instance, *Smad1* and *Smad5*, normally expressed in the epiblast during gastrulation, are required for PGC specification (Chang and Matzuk, 2001; Tremblay et al., 2001; Hayashi et al., 2002). Both *Smad1*- and *Smad5*-deficient embryos develop with reduced number of PGCs. In addition, PGC specification requires heterodimers that *Smad4* forms with either *Smad1* or *Samd5* to transduce

the *Bmp* signal (Chu et al., 2004). Taken together, the *Bmp* signals are essential for PGC specification. However, the mechanism by which these signals induce differentiation of only a subset of posterior proximal epiblast cells into PGCs largely remains unknown.

Genes involved in PGC specification

Despite the significance of embryological origin of PGCs and their dependency on the *Bmp* signaling pathway, genes involved in specification and maintenance of PGCs remain poorly understood. Because a smaller number of their founder population exists and is embedded deeply in somatic cells, analyzing gene expression during establishment of PGCs is a challenge. On the other hand, by means of single cell gene expression analysis, Saitou et al. (2002) has demonstrated that PGCs with high levels of an interferon-inducible trans-membrane protein *fragilis/mil-1/IFITM3* (Saitou et al., 2002; Tanaka et al., 2005) and with exclusive expression of a small nuclear-cytoplasmic shuttling protein *stella/PGC7/Dppa3* (Saitou et al., 2002; Sato et al., 2002; Bortvin et al., 2003) repress *Hoxb1* and *Hoxa1*, whereas these genes are highly up-regulated in somatic neighbor cells (Saitou et al., 2002). Therefore, PGCs may escape from a somatic cell fate and commit to the germ-line by repressing expression of these homeobox genes. Additionally, the founder population of PGCs as well as their somatic neighbors express mesodermal markers *T/ Brachyury* and *Fgf8* (Saitou et al., 2002). Thus, PGCs are specified from a population of cells originally destined for the mesodermal fate.

Further, single cell analysis has identified that *Blimp1/Prdm1* (B lymphocyte-induced maturation protein-1) is expressed in the founder population of PGCs (Turner et al., 1994; Ohinata et al., 2005). *Blimp* is a zinc-finger-containing DNA-binding transcriptional repressor, functions as a master regulator of B cell terminal differentiation. It has also been shown to

repress the expression of cell cycle regulators such as *Myc*, p18 and p21 and to downregulate the expression of other transcription factors, playing important roles in various aspects of embryonic development in many organisms (Vincent, 2005; Yang, 2005; Martins, 2008). In *Blimp1* mutant mouse embryos, the number of alkaline phosphatase (AP) -positive cells that are indicative of being PGCs was reduced at the early bud and early head fold stages of late E7.5 embryos, and was not increased in subsequent embryonic development. These AP-positive cells in *Blimp1* mutant embryos failed to migrate, consistently repress *Hox* genes, and express genes specific for PGCs, including *stella* (Ohinata et al., 2005). Therefore, these findings indicate that *Blimp1* plays a central role in PGC specification (Ohinata et al., 2005). However, the underlying interaction among *Bmp* signaling, *Blimp1*, *Fragilis* and *Stella* in specification, migration and proliferation of PGCs remains to be investigated.

Molecules involved in PGC migration

After being specified, PGCs form a tight cluster at the base of the allantois and initiate their migration individually toward the future genital ridge at around 8 days after fertilization (Tam and Snow, 1981; Anderso et al., 2000). A number of genes play important roles for migration, proliferation and maintenance of PGCs.

Genetic studies have shown that the proto-oncogene receptor tyrosine kinase *c-kit* and its ligand the *Steel factor (Sl)* are necessary for migration, survival and proliferation of PGCs (Nocka et al., 1989; Godin et al., 1991; Besmer et al., 1993; Miguel et al., 2002). Additionally, evolutionarily conserved chemokine Stroma cell-derived factor1 *(SDF1)/CXCL12* and its G-protein-coupled receptor *CXCR4* are crucial for migration of PGCs (Molyneaux et al., 2003).

The POU domain transcriptional factor *Oct3/4*, which is expressed in pluripotent cells of preimplantation embryos and embryonic stem cells (Scholer et al., 1990; Yeom et al., 1996), is also necessary for the survival of PGCs, because PGC-specific deletion of *Oct3/4* results in apoptosis of PGCs (Kehler et al., 2004). However, it remains to be determined how *Oct3/4* deficiency leads to apoptosis of PGCs. Because another key transcription factor *Sox2* collaborates with *Oct3/4* to maintain cellular pluripotency (Masui et al, 2007), it will be critical to study the function of *Sox2* in maintenance of PGCs.

Interestingly, it has been recently shown that Hypoxia-inducible factor2α (*Hif2α*) plays a crucial role in specification of PGCs through regulating transcriptional activation of *Oct3/4* in PGCs (Covello et al., 2006). In *Hif2α* mutant mice, the number of AP-positive PGCs was dramatically decreased 8 days after fertilization (Covello et al., 2006). *Hifs* are the main transcriptional regulators necessary for systemic hypoxic adaptation of mammalian cells (Semenza, 2000; Keith and Simon, 2007). *Hifs* regulate expression of at least 180 genes involved in metabolism, erythropoiesis, vascular remodeling, and cell survival (Semenza, 2000). However, it remains to be elucidated whether *Hif2α* directly binds to the regulatory region of *Oct3/4* in the PGC precursors.

In mice, the *Ter* mutation causes PGC loss by 8 days after fertilization when PGCs start to migrate and proliferate (Stevens, 1973; Noguchi and Noguchi, 1985). No matter how the Ter allele is retained, the *Ter* mutation is dominant in all genetic background (Sakurai et al., 1995). Recently, the *Ter* mutation was defined as a point mutation in the gene encoding a mouse orthologue of the zebrafish dead end gene (*Dnd1*), which introduces a premature termination codon in *Dnd1* (Youngren et al., 2005). However, the underlying mechanism by which *Dnd1* regulates PGC migration and proliferation remains unknown.

Nanos encodes an RNA-binding protein and plays a critical role in germ cell development and abdominal patterning in *Drosophila* (Wang and Lehmann, 1991; Subramaniam and Seydoux, 1999; Tsuda et al., 2003). *Nanos* suppresses gene expression required for differentiation of somatic cells. In the absence of maternal *Nanos*, PGCs failed to migrate into gonad and were not able to differentiate into functional gametes (Kobayashi et al., 1996; Hayashi, et al, 2004). In mice, expression of the mouse homologue of *Nanos*, *Nanos3*, is found in PGCs as early as 7.25 days after fertilization (Yabuta et al., 2006). In the absence of *Nanos3*, PGC specification occurs normally but the number of PGCs is reduced 8.0 after fertilization, and all the PGCs are lost eventually (Tsuda et al., 2003). However, the underlying mechanism by which Nanos3 regulates maintenance and proliferation of PGCs remains to be explored.

Taken together, many cell signaling molecules, RNA-binding proteins and transcription factors play essential roles in PGC migration, proliferation and maintenance/survival. However, the direct interactions of these molecules as well as the underlying mechanism in which these molecules are involved remain unexplored. Needless to say, other gene product(s) may play a similar role in specification, migration, proliferation and/or maintenance of PGCs.

Pluripotency of PGCs

When PGCs aberrantly migrate elsewhere than the genital ridges, such aberrant PGCs normally initiate the cell death program due to the lack of survival factors such as *Sl* and/or *CXCL12* (Godin et al., 1991; Besmer et al., 1993; Molyneaux et al., 2003; Runyan et al., 2009). However, occasionally these aberrant PGCs grow as tumors called teratomas which are benign monstrous tumors containing a variety of differentiated cells of ecto-, meso- and endoderm origin (Stevens, 1967). Despite this fact, when PGCs are isolated and mixed with

preimplantation embryos, PGCs do not contribute to the body of chimeric embryos (Donovan, 1998). On the contrary, when cultured *in vitro* in the presence of exogenous signaling molecules, such as fibroblast growth factor2 (*FGF2*), leukemia inhibitory factor (LIF) and Steel factor/stem cell factor (*Sl/SCF*) (Durcova-Hills et al., 2006; Durcova-Hills et al., 2008), PGCs can become pluripotent stem cells similar to embryonic stem cells, namely embryonic germ (EG) cells. EG cells can differentiate into many specialized cell types *in vitro* and can contribute to development of normal chimeric embryos after injection into blastocysts (Zwaka and Thomson, 2005). Thus, PGCs are potentially pluripotent in a context dependent manner (Durcova-Hills et al., 2006). It would be interesting to study the mechanism by which PGCs can be reprogrammed into pluripotent stem cells in vitro. This exact mechanism may also function in an opposite way to prevent PGCs from aberrantly becoming pluripotent and differentiating in ectopic places.

Remarkably, it was demonstrated by a stunning study in 2006 that, with retroviral transduction of four transcription factors, *Oct3/4, Sox2, Klf4* and *c-Myc*, mouse adult fibroblasts were able to be reprogrammed into pluripotent stem cell-like cells similar to embryonic stem (ES) cells, and these cells were named induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). Subsequently, human iPS cells have been generated using transcription factors *Oct3/4* and *Sox2,* and additionally with either *Klf4* and *c-Myc* (Takahashi et al. 2007) or *Nanog*, and *Lin28* (Yu et al., 2007), which making an unprecedented opportunity to create patient-specific disease models for research purposes possible, ESCs are considered to be equivalent to the inner cell mass (ICM) cells because ESCs are derived from them. However, emerging evidences suggest that the *in vivo* counterpart of ESCs is PGCs (Zwaka and Thomson, 2005). Interestingly, PGCs are the only cells in developing embryos that express three of the key factors to generate iPS cells, *Oct3/4, Sox2* and *Nanog*, after gastrulation (Yabuta et al., 2006). It

remains to be investigated if PGCs express *Klfs* or *c-Myc*. Furthermore, it has recently been shown that putative PGCs can be derived from human ESCs by making modest changes to the growth conditions commonly used to expand hESCs, without using genetic manipulation or complex three-dimensional culture (Bucay et al., 2009). However, a question remains: how cells expressing key genes required for the reprogramming become pluripotent stem cells? Further studies on germ cell differentiation and the pluripotent potential of PGCs may lead us to a better understanding of the reprogramming event.

Rhox **Gene Family**

Transcription factors play an essential role in altering gene expression. It has been estimated that 10% of proteins within a cell are transcription factors that regulate important cellular processes, including cell lineage determination, differentiation, cell growth, and temporal or cell type-specific gene expression (Lemon and Tjian, 2000). Therefore, studies focusing on detailed interpretation of the regulatory role of transcription factors provide a better understanding of how various aspects of development events are achieved through precise transcriptional control.

Homeobox genes encode transcription factors containing a 60-amino-acid DNA binding domain called homeodomain (Gehring and Hiromi, 1986). The homeodomain was first identified because mutations in the genes encoding such a protein domain commonly resulted in homeotic transformation of *Drosophila* segments (McGinnis and Krumlauf, 1992; Gehring et al., 1994). Later, genes encoding the homeobox are widely found among metazoan species analyzed, ranging from sponges to vertebrates (Carrasco et al., 1984) including humans (Levine et al., 1984), and also in plants (Ruberti et al., 1991; Vollbrecht et al., 1991) and fungi (Shepherd et al., 1984; Schulz et al., 1990). The homeobox allows homeodomain proteins to recognize the

specific target sequences. Therefore, these homeodomain proteins act as transcription factors that regulate expression of their target genes in a spatially and temporally coordinated manner (Gehring et al., 1994). The homeodomain transcription factors regulate many developmental processes, including axis formation, limb development, and organogenesis (Weatherbee et al., 1998; Kmita and Duboule, 2003).

There are over 50 homeobox genes expressed in the male reproductive organs (Shashikant et al., 1991; Hu et al., 2007). However, their precise functions in spermatogenesis and fertility remain largely unknown (Branford et al., 1997; Li et al., 1997). By contrast, recently, a new cluster of 12 homeobox genes has been indentified on the X chromosome of the mouse. They are specifically expressed on male and female reproductive organs, such as the testis, epididymis, ovary, and placenta (MacLean et al., 2005), playing important regulatory roles in reproduction. Thus, they are named Reproductive Homeobox on the *X* chromosome, *Rhox*, and are numbered based on their physical distance from the centromere.

A member of *Rhox* family, *Rhox5,* is originally named *Pem* (Sutton and Wilkinson, 1997) and is expressed in PGCs (Daggag et al., 2008) and the placenta (MacLean et al., 2005). Furthermore, *Rhox5* is expressed in Sertoli cells during spermatogenesis in mature male mice (Bhardwaj et al., 2008). It has been shown that targeted disruption of *Rhox*5 resulted in increased male germ cell apoptosis and reduced number of sperm, sperm motility and fertility (MacLean et al., 2005), although *Rhox5*-deficient mice are fertile (Pitman et al., 1998). The result support the idea that *Rhox* play important roles in reproduction.

Another member of *Rhox* family, *Rhox9,* is expressed in the germ cells in developing gonads and the placenta (Han et al., 2000; Takasaki et al., 2000). Also, *Rhox9* is expressed in both male and female embryonic urogenital ridges at the onset of sexual dimorphism in mice, with more

transcripts accumulated in female germ cells in later embryonic development (Takasaki et al., 2000). *Rhox9* is originally named *Psx2*, and has been demonstrated to have no significant biological function, even in reproduction (Takasaki et al., 2001). However, this observation may raise a possibility that other members of the *Rhox* family can functionally compensate the deficiency in the *Rhox9* function.

To support this idea, another member of the *Rhox* family, *Rhox6*, is highly homologous to *Rhox9* and 80% of its homeodomain is identical to that of *Rhox9* (MacLean et al., 2005). *Rhox6* is originally named *Placenta-specific homeobox 1 (Psx1)* and expressed specifically in the placenta, but not detected in fetus, pups, and adult tissue(Han et al., 1998; Chun et al., 1999; Tanaka et al., 2000). Similar to the expression pattern of *Rhox9*, *Rhox6* is also expressed in the male and female embryonic urogenital ridges at the onset of gonadal dimorphism (Takasaki et al., 2000; Daggag et al., 2008). The comparable levels of *Rhox6* are initially present in both sexes, and its transcripts accumulate more abundantly in female germ cells in later embryonic development (Takasaki et al., 2000; Daggag et al., 2008). So far, there is no study about *Rhox6*-deficient mice reported.

The Embryonic Stem Cell as A Powerful Tool to Study Biological Functions of Genes in Germ Cell Differentiation

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of blastocysts when blastocysts are cultured *in vitro* (Evans and Kaufman, 1981; Martin, 1981). Those cells are considered pluripotent cells as they maintain the ability to undergo differentiation to almost all of the cell types *in vitro* and they participate in embryonic development when injected into blastocysts ESCs (Bradley et al., 1984). There are three main advantages of ESCs as a surrogate

assay system to study embryogenesis; (1) they can provide a source of relatively uniform undifferentiated cells, (2) they can provide an unlimited number of cells, and (3) they can provide mutant models. Due to these reasons, ESCs serve as one of the primary tools to understand the mechanisms of cell fate decision (Giadrossi et al., 2007; Nishikawa et al., 2007).

Primordial germ cells (PGCs) are closely related to embryonic stem (ES) cells and embryonic germ (EG) cells (Zwaka and Thomson, 2005). Mouse ESCs can differentiate into PGCs, and gametes *in vitro* (Hubner et al., 2003, Geijsen et al., 2004). Recently, immature sperm cells derived from mouse ESCs in culture have generated live offspring (Nayernia et al., 2006). Although still preliminary, a recent study has demonstrated that human ES cells (hESCs) apparently display a similar developmental capacity to generate PGCs and immature gametes (Bucay et al., 2009). Therefore, this ES cell technology offers great potential for new types of reproductive biological studies including a readily accessible system to understand germ cell development in mouse as well as in human.

ESCs can differentiate into the three germ lays through the use of specific culture conditions, such as embryoid body (EB) formation (Doetschman et al., 1985), co-culture on Stroma cells (Nakano et al., 1994) and culture on matrices (Nishikawa et al., 1998). Currently, the most popular protocol to efficiently induce differentiation of ESCs is to make EBs, in which a variety of cell types differentiate spontaneously (e.g. Nishikawa et al., 2007). Forming EBs with ESCs is to mimic the gastrulation process in developing embryos (Nishikawa et al., 2007). Among a handful of developmentally important signaling pathways, the *Wnt* pathway mediates the local execution of a gastrulation-like process in the EB (ten Berge et al., 2008). However, differentiation of different cell types in EBs is a random event. To enrich a cell type of interest, ESCs are often genetically modified to express a reporter gene such as the enhanced green

fluorescent protein (EGFP) under the regulatory sequence of a gene that is specifically expressed in the cell type of interest. Thus the desired cell type can be purified. Therefore, forming EBs with genetically modified ESCs is a convenient approach to induce cell differentiation, which provides a surrogate assay system for developmental biological analysis (e.g. Nishikawa et al., 2007).

During the past several years, RNA interference (RNAi) has emerged as a powerful gene-knockdown technology in many model eukaryotic systems. Short hairpin RNAs (shRNAs), one of the RNAi technologies, is becoming increasingly important in functional genetic studies in cell and developmental biological studies (Schaniel et al., 2006). shRNAs are processed by Dicer to form a small interfering RNA (siRNA), which is then incorporated into the RNA-induced silencing protein complex (RISC). The RISC functions to unwind the siRNA duplexes to allow specific binding to the complementary mRNA target, followed by siRNA-mRNA duplex cleavage (Rutz and Scheffold, 2004; Cullen, 2005). At the end, RISC is released from the target mRNA and the cleaved mRNA is further degraded by cellular exonucleases (Holen et al., 2002). Therefore, the specific degradation of the target mRNA results in decreased synthesis of the respective protein and eventually leads to loss of the protein function (Rutz and Scheffold, 2004). Collectively, in combination with shRNA technology, ESCs are considered as a powerful tool to study gene functions during cell differentiation events, including germ cell differentiation.

CHAPTER 2

Rhox6 is required for testis development and spermatogenesis in mouse

Abstract

Homeodomain proteins are responsible for a variety of developmental events such as determination of body segment identity, limb development and organogenesis. Recently, a novel cluster of homeobox genes has been identified on the X chromosome of the mouse. Because of their selective expression in reproductive organs, they are named *Reproductive homeobox genes on the X chromosome (Rhox*).

Rhox6, a member of the *Rhox* family, is expressed in primordial germ cells (PGCs) in the developing gonads and the placenta, although the biological function of *Rhox6* in the determination of PGCs and placental cells remains unknown. To investigate the *Rhox6* function, we generated male chimeras by injection of an embryonic stem cell line (ESCs) harboring a hypomorphic mutation in *Rhox6* into wild-type blastocysts. Almost all of the male chimeras exhibited reduced fertility associated with smaller testes, reduced sperm number, sperm motility and abnormal morphology of the epididymal sperm. Therefore, the results indicate that downregulated expression of *Rhox6* impaires testis development and spermatogenesis in mice.

Introduction

Spermatogenesis is a complex process that involves renewal of germ-line stem cells and differentiation of the sperm, which further includes a cascade of events such as recombination of the genome and condensation of chromosomes. Therefore, problems occurring at any stage of spermatogenesis may contribute to infertility. However, currently there are only a few *in vitro* systems available, which can recapitulate spermatogenesis and serve as a model to study infertility. By contrast, targeted mutagenesis in the mouse has been providing useful models to study gene functions in spermatogenesis, which has brought new insights into causality of male infertility (Cooke and Saunders, 2002).

A member of the *Rhox* family, *Rhox6,* is expressed in primordial germ cells in the developing gonads and the placenta (Han et al., 1998; Takasaki, 2000; Daggag, 2008). Predominant expression of *Rhox6* in the mid-gestation placenta was independently validated by the earlier study conducted in our laboratory (Tanaka et al., 2000) . One of our research interests is to understand the genetic mechanism of the first cell differentiation event that segregates the extraembryonic cell lineage from pluripotent cells in preimplantation embryos. Further, despite its unique expression pattern, significance of *Rhox6* expression in development of the extraembryonic cell lineage remains unknown. Therefore, to investigate the biological function of *Rhox6* in differentiation of the extraembryonic cells, we generated chimeras by injecting a genetically-modified embryonic stem cell line, which harbors a hypomorphic mutation in *Rhox6*, into wild-type blastocysts. Our results suggest that *Rhox6* may play a crucial role in differentiation of the testis and spermatogenesis.

Materials and Methods

Generation of chimeras

The embryonic stem cell line that harbors the hypomorphic mutation in *Rhox6* was generated by randomly insertional mutagenesis, namely gene trap. Single pass-sequencing using the Neomycin resistant gene as a reference was carried out by the Center for Modeling Human Disease in Toronto, Ontario, Canada which identified that the 80.1H12 ES cell line harbors insertion of the gene trap cassette between Exons 2 and 3 of the *Rhox6* gene (Fig. A1). The gene trap cassette contains the promoter-less β-galactosidase gene. Chimeras were generated by injecting the 80.1H12 ES cell line into the blastocoels of wild-type (C57BL/6) blastocysts as described (Joyner, 1999). Because 80.1H12 has the male karyotype, male chimeras were kept for mating to transmit the mutant *Rhox6* allele into next generations.

For mating, each chimera was caged with a wild-type C57Bl/6 female for three days. The presence of the vaginal plug was checked every day. Male chimeras were then rested for a week before starting a next mating. The mating was conducted for 2~3 months.

Analysis of sperm motility, sperm count, and abnormality

Sperm was collected from the cauda epididymis of both chimeric and wild-type mice and analyzed essentially as described previously (Larson and Miller, 1999). Briefly, the cauda epididymis from each side of the mouse was poked and squeezed using a 30-G needle to expel the sperm in the Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 15% fetal bovine serum (FBS; Invitrogen), glutamax I (Invitrogen), and penicillin-streptomycin (Sigma), and incubated for 10 min at 37^{o}C to allow the sperm to regain their activity. The sperm

suspension was placed on a glass-slide to check motility of the sperm under a standard microscope. The number of the sperm was counted with a hemocytometer.

The sperm collected from the cauda epididymis were fixed with 2% paraformaldehyde solution for overnight. The fixed sperm was then centrifuged and washed twice with 100mM ammonium acetate (pH 9.0), followed by resuspension into into 1ml of 100mM ammonium acetate. The sperm suspension was smeared on glass slides, air-dried, and stained with Coomassie brilliant blue G-250 for 2 min followed by washing with distilled water. After air-drying these slides were mounted with Permount mounting medium for microscopic examination.

Results

The male chimeras generated with the 80.1H12 ESC line were sterile

Chimeras were generated by injecting a mutant embryonic stem cell line of the 129 background, which is named 80.1H12 and harbors a hypomorphic mutation in *Rhox6*, into wild-type C57Bl/6 blastocysts. Because *Rhox6* is highly expressed in the placenta, the *Rhox6* mutant embryos were expected not to implant when the hypomorphic mutation was transmitted into the next generation from the chimera.

Interestingly, however, all of the male chimeras showed seriously reduced fertility or infertility. When ESCs are infected with mycoplasmas or possess abnormal karyotypes, these ESCs do not differentiate into germ cells. However, we ruled out these possibilities because the 80.1H12 ESCs have a normal karyotype and no cytoplasmic DNA stain which is indicative of mycoplasma contamination. Therefore, the infertility observed in these male chimeras is due to contribution of the 80.1H12 ESCs.

There are two independent attempts to generate chimeras. In the first attempt we obtained two male chimeras with different degrees of chimerism, which are named as 20% chimera and 60% chimera based on their *Agouti* coat color attributed from the 80.1H12 cell line. For instance, 20% chimera means that *Agouti* accounts for 20% of the coat of this chimera, although it only indicates that the skin cells derived from the 80.1H12 ESCs accounts for 20% of total skin cells in this chimera.

The 20% chimera made one female pregnant out of 3 mating attempts. That female delivered 5 pups. However, according to the coat color, all pups were wild-type, indicating that the *Rhox6* mutation could not be transmitted to the next generation. On the other hand, no female became pregnant by mating with the 60% chimera out of 6 attempts, which indicates that 60% chimera was infertile. In the second attempt to generate chimeras with the 80.1H12 ES cell line, we obtained two chimeras named as 90%A and 90%B. For the 90%A chimera, no female became pregnant out of 8 mating attempts. The 90%B chimera made two females pregnant out of 7 mating attempt, delivered 2 and 4 pups with black coat color respectively.

The male chimeras showed tendency to produce fewer sperm with reduced motility, which was correlated with their testis size.

We collected epididymal sperm from the chimeric and wild-type mice and analyzed their motility and the number. We found that nearly all the chimeras showed decreased number of sperm and reduced motility compared to the wild-type (Table 2.2).

The sperm from the 20% chimera exhibited normal motility, whereas the number of its sperm was reduced to 72% of that from wild-type mice (Table 2.2). However, for the 60% chimera, the number of the sperm collected from the epididymis extending from the testis of normal

appearance was only 13.3% of that from wild-type mice and 50% of the sperm were motile. Furthermore, 10% of the epididymal sperm obtained from the smaller testis of the 60% chimera were motile, and the number of its epididymal sperm was only 2.4% of that from wild-type mice (Table 2.2.). Therefore, this result indicates that the percentage of contribution of the *Rhox6*-mutant ESCs in the coat color may correlate with that in the testis and that the higher the contribution of the *Rhox6*-mutant ESCs is, the more sever the reduction in the number and motility of the sperm in the chimeras is. Likewise, in the second attempt to generate chimeras, the degree of defects observed in the number and motility of the epidydimal sperm from chimeras were consistent with the reduction in their testis size. At most 10% of the epididymal sperm from the 90%A chimera, which had two smaller testes, were motile, and the number of its epidydimal sperm was less than 1% of that of wild-type mice. On the other hand, at least 50% of the epididymal sperm from the 90%B chimera, which had two testes of normal appearance, were motile and the number of its epididymal sperm was 88% of that from the wild-type mice (Table 2.2).

The male chimeras showed tendency to produce abnormal sperm which was correlated with their testis size.

To evaluate abnormality of the sperm collected from the cauda epididymis of chimeras, morphology of their heads and tails was examined by staining with Coomassie brilliant blue G-250 (Larson and Miller, 1999). Because normal sperm exhibit a series of defects in general (Dadoune et al., 1994), I categorized these defects into four types arbitrarily: Type I, sperm with cytoplasm droplets; Type II, sperm without the acrosome; Type III, sperm with folded tails,

which include the angled tails and hairpin forms; and Type IV, sperm with abnormal or missing heads (Fig 2.2).

Almost all of the Type I sperm are ones with the droplet between the middle piece and the sperm tail, which is a minor defect and do not have a serious impact on fertility (Kawai et al., 2006). Normally, these droplets are removed when sperm mature (Kawai et al., 2006). The 20% chimera had a higher percentage of the type I sperm (~20% of the total sperm from the 20% chimera) relative to the other chimeras. One the other hand, about 30% of the wild-type sperm were classified as the type I sperm (Fig. 2.3). Therefore, it is highly unlikely that the presence of the *Rhox6*-mutant ESCs in the chimeras caused this abnormality. At the expense of this type of sperm abnormality, the chimeras exhibited much more sever sperm abnormality as described below.

The type II sperm existed at a similar frequency among the chimeric and wild-type mice examined. Thus, the presence of the *Rhox6*-mutant ESCs in the chimeras is less likely to contribute to the missing acrosomes.

In general, the type III sperm results in decreased fertility (Kawai et al., 2006). In particular, *spem1*-deficient mice exhibit this type of sperm abnormality due to the aberrant cytoplasm removal during sperm maturation (Zheng et al., 2007). The 60% chimera had a highest percentage of the type III sperm among the chimeras. About 20% to 40% of the epididymal sperm from the 20%, 90%A and 90%B chimeras were the type III sperm whereas about 10% of the wild-type sperm had the type III sperm.

Generally, a defect in spermatogenesis results in generating the type IV sperm , and mice with a higher percentage of the type IV sperm are sterile (Meistrich et al., 1994; Mendoza-Lujambio et al., 2002). I found that the 90%A chimera that had two significantly smaller testes

had the highest percentage of the type IV sperm in its epididymis. Likewise, about 22% of the epididymal sperm collected from the smaller testis of the 60% chimera was the type IV sperm. Consistently, the larger the size of the testis is, the less the frequency of the type IV sperm in the epididymal sperm is.

The 20% and 90%B chimeras had a similar frequency of the sperm with normal appearance in the epididymis (~20%), whereas more than 30% of the epididymal sperm from the wild-type mice were normal appearance. The smaller the testis size of the chimeras is, the less the frequency of the sperm with normal appearance is. .

Despite the fact that the 60% chimera had one testis of normal appearance and the other smaller one, its epidydimal sperm consistently had a higher frequency of the type III sperm, which was even the highest observed among the chimeric and wild-type mice examined. By contrast, the epididymal sperm from the 90%A chimera that had much smaller testes had the higher percentage of the type IV sperm, which was the highest observed among the chimeric and wild-type mice. Defects in spermatogenesis result in generating more type III and type IV sperm (Yeung et al., 1999). Collectively, these results indicate that the presence of *Rhox6*-mutant ESCs in the chimeric testis interferes with normal spermatogenesis.

Discussion

Consistently, the phenotype of the chimeras was highly correlated with the reduced number and motility of sperm and defects indicative of abnormal progression of spermatogenesis. I observed one smaller testis in the 60% chimera and two smaller testes in the 90%A chimera. Interestingly, I found that the testis on the left side of the 90%A /chimera was extremely small (Fig. 2.1-D). In the 60% chimera, the epididymal sperm collected from the normal testis had the

largely reduced number of sperm (13.3% of that of the wild-type mice) and showed lower motility. The number of the epididymal sperm obtained from the other smaller testis was smaller (2.4% of the wild-type; Table 2.2.). This percentage was far smaller than I expected because, if the contribution of mutant ESCs observed in the coat color indicates that in the chimeric testis, 40% of cells in the testis should be wild-type cells. Likewise, the number of the epididymal sperm collected from the 90%A chimera is less than 1% of that of the wild-type mice. Therefore, these data indicate that differentiation of the wild-type germ cells in the chimeric testes might also have been impaired by the presence of the *Rhox6*-mutant ESCs.

One of the reasons for the abnormal phenotype in the chimeric testes can be deformation of seminiferous tubules. It remains to be examined if *Rhox6* or its downstream target(s) affects differentiation of somatic cells in the testis such as peritubular cells (Clark et al., 2000).

The percentage of hairpin sperm and sperm with severely folded tail (20%) in the epididymal sperm collected from the right cauda epididymis (Fig 1-C) from the 90%A chimera was less than the percentage of similar abnormalities (31%) in the sperm from the left (Fig 1-D). By contrast the percentage of the normal sperm (13.24%) in the epididymal sperm collected from the right side of the 90%A chimera is higher than that from the left side (7.59%). Because the testis in the left side of the 90%A chimera had a much smaller testis, the increased proportion of the abnormal sperm shows good agreement with the size of the testis observed. However, it remains to be investigated if spermatogenesis in the chimeras was affected by the putative defects in development of seminiferous tubules due to the presence of the *Rhox6*-mutant ESCs affects.

Besides, further investigation of chimerism in testes would help understand how testis development and spermatogenesis are affected by the presence of cells that have reduced *Rhox6* expression.

Figures and Tables

Table 2.1 Summary of mating history of chimeras.

Chimera	**Times of mating**	**Plug**	**Times of Pregnancy**	**Number of pups**	**Coat color of pups**
20% chimera	3	-	1	5	Black
60% chimera	6	-	-	-	-
90%A chimera	8	-	-	-	-
90%B chimera	7	-	2	2	Black
				4	Black

"-": None

Table 2.2: Summary of abnormality in the chimeras

Chimera	**Testis size**	**Sperm motility**	**Sperm count** ***(x 10^7/ml)***
Series I			
20% chimera	N_1	80%	2.0
(5 month old)	N_2	80~90%	1.9
60% chimera	S	10%	0.065
(5 month old)	N	50%	0.36
F1 (W.T.)	N_1	80~90%	2.6
(5 month old)	N_2	80~90%	2.8
Series II			
90%A	S_1	10%	0.023
(8 month old)	S_2	<5%	0.004
90%B	N_1	45%	2.375
(8 month old)	N_2	70%	2.35
C57BL6 (W.T.)	N_1	80~90%	3.375
(8 month old)	N_2	90%	3.4

S: smaller ; N: normal ; F1 (W.T.) and C57BL6 (W.T.) serve as control.

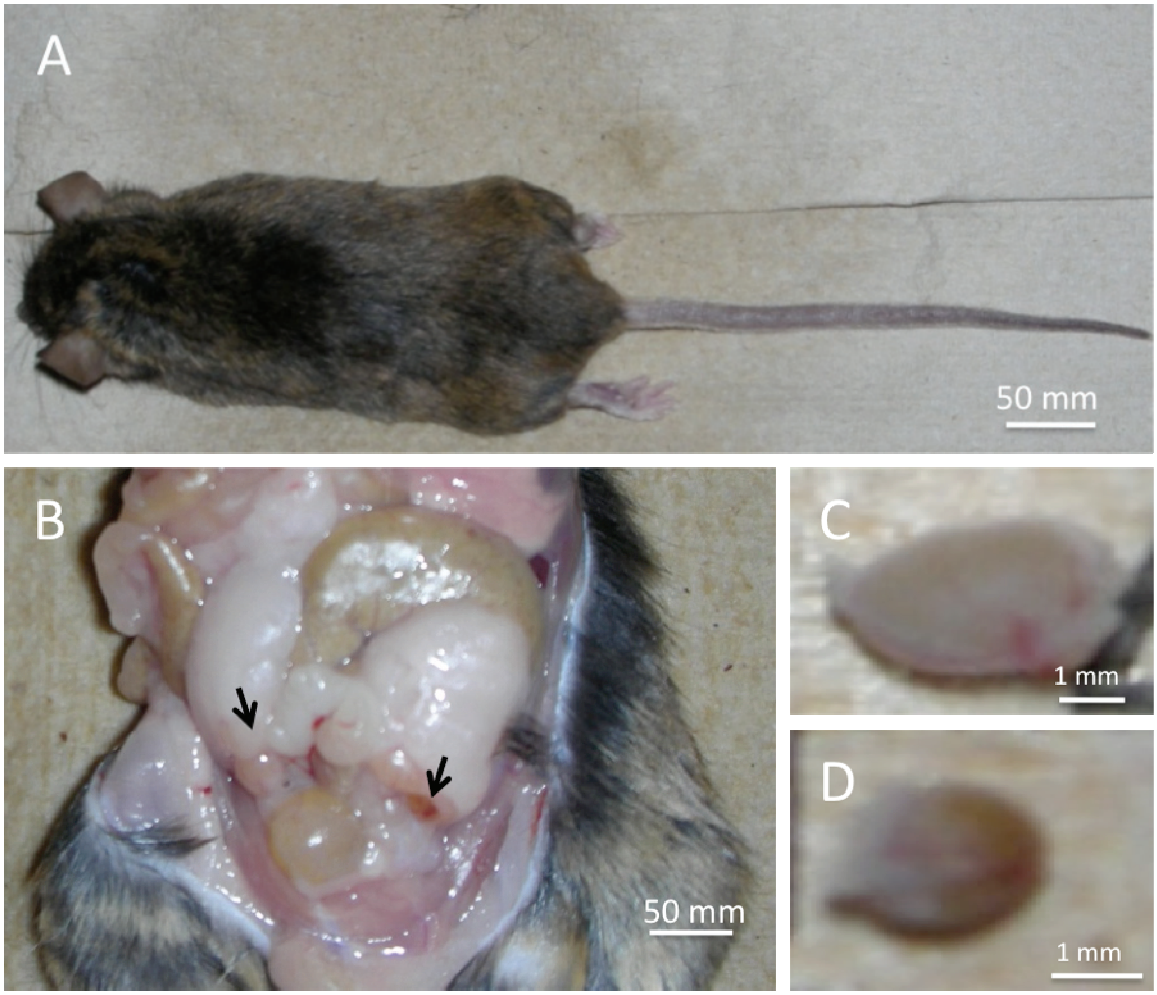


Fig.2.1 (A,B) A representative male chimera with its gross anatomy including testes (arrow) is shown. Based on the *Agouti* coat color, this chimera is termed as "90%A chimera". (C) The right testis of "90%A chimera" mouse. (D) The left testis of "90%A chimera" mouse.

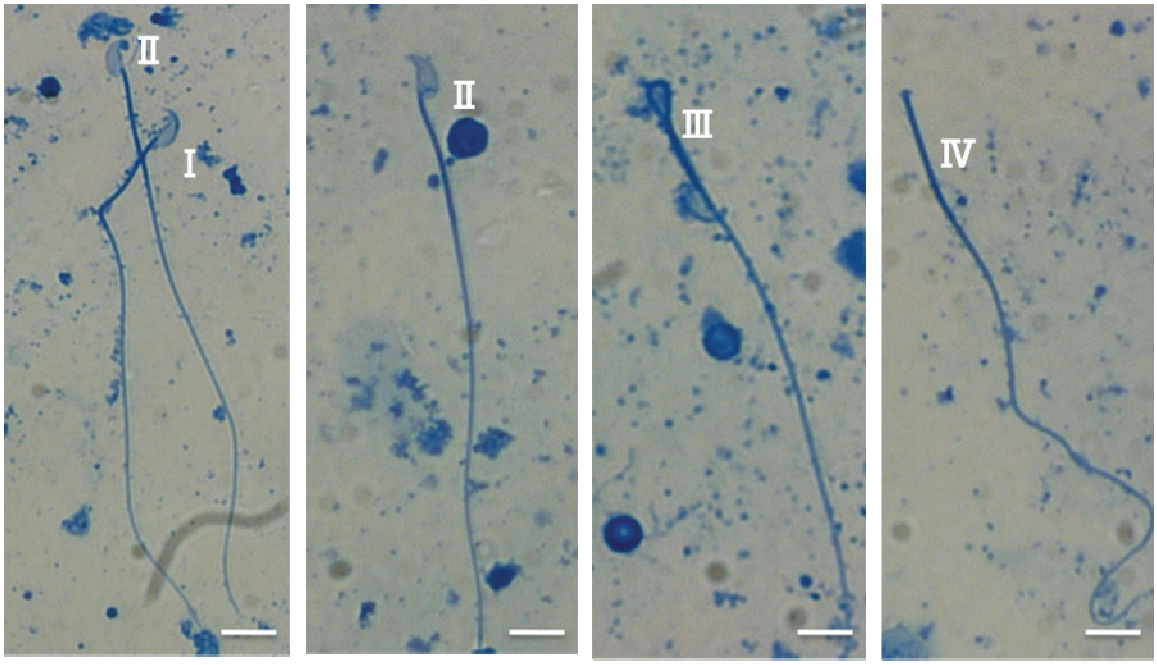


Fig. 2.2: representative abnormalities of sperms. I: normal sperm; II: sperm without acrosome; III: sperm with folded tail (hairpin); IV: sperm without head. Scale Bar: 50µm.

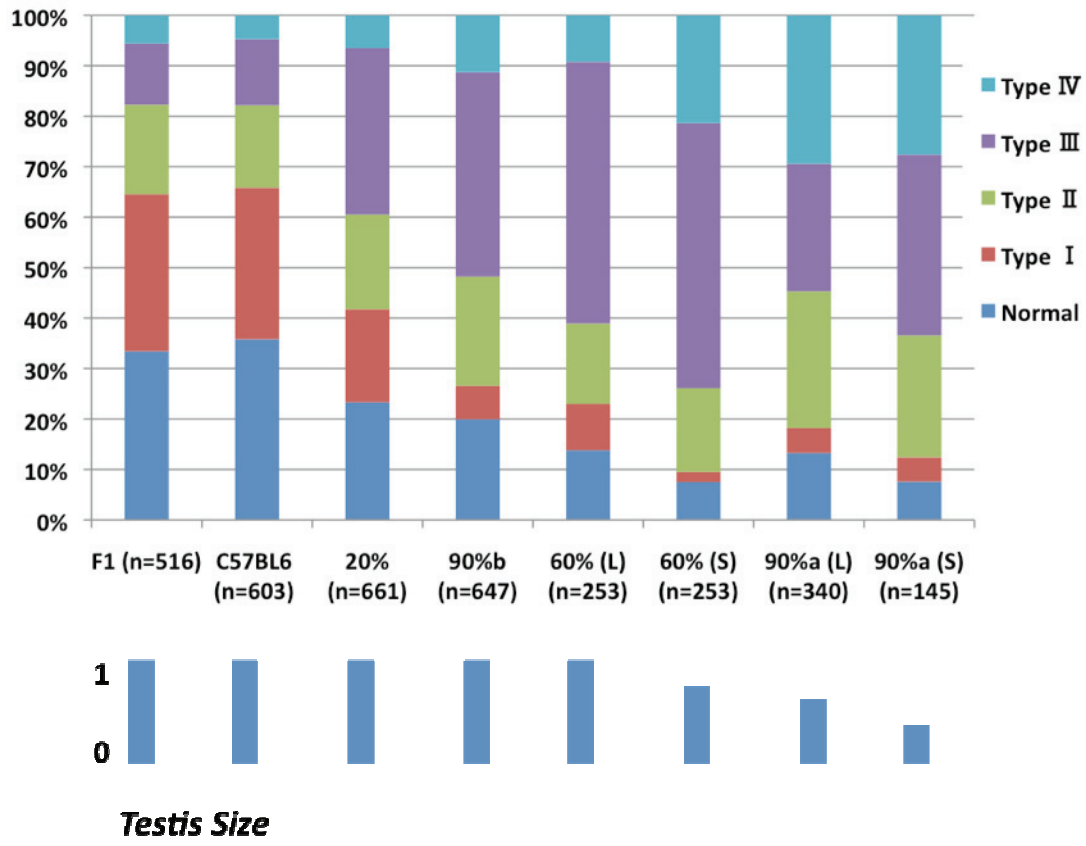


Fig 2.3. Abnormality of the sperm from the wild-type, the 20% chimera, the 60% chimera, the 90%A chimera and the 90%B chimera. Type I, sperms with cytoplasm droplets either between sperm head and middle piece or between the middle piece and sperm tail; Type II, sperms without acrosome; Type III, sperms with folded tails, which included the angled tail and hairpin forms; Type IV, sperms with abnormal head or missing head; Normal, normal sperms. The figure is organized in order of testis size. L: larger testis; S: smaller testis.

CHAPTER 3

Rhox6 is required for differentiation of germ cells from embryonic stem cells

Abstract

In the previous chapter, it is shown that *Rhox6* is required for normal testis development and spermatogenesis. To obtain a better understanding of the *Rhox6* function in germ cell differentiation, I took advantage of *in vitro* ES cell culture. ES cells can differentiate into PGCs when ES cells are cultured as aggregates called embryoid bodies (EBs) with the culture medium that promotes differentiation of ESCs. Because PGCs express the transcription factor *Oct3/4*, a central regulator of cellular pluripotency, buse of a mouse ESC line that expresses the enhanced green fluorescent protein (EGFP) under the promoter of *Oct4* allows us to monitor the presence of PGCs within EBs in real time. While this ESC line was induced to differentiate into PGCs, expression of *Rhox6* was downregulated by stable expression of the short-hairpin RNA (shRNA) against the *Rhox6* transcripts. The level of *Rhox6* knockdown was validated by RT-PCR. As a negative control, a scrambled nucleotide sequence that does not show any match with the mouse genome was used to express shRNA molecules.

Multiple independent ES cell clones that express either one of the shRNAs were isolated by electroporation of the *Oct3/4*::EGFP-ESC line with each expression vector for the shRNA, followed by drug selection. These ES cell clones were used to generate EBs. We found that 2% of the EBs having the shRNA against the *Rhox6* transcripts expressed (n=42; the number of clones used to generate EBs is 27) had EGFP-positive cells, whereas 41% of the EBs having the control shRNA expressed (n=48; the number of clones used to generate EBs is 31) had EGFP-positive cells. To validate differentiation of PGCs, these EBs were pooled according to EGFP expression and used to examine expression of PGC markers by semi-quantitative RT-PCR.

Because expression of *Rex1*, a marker for undifferentiated ESCs, was significantly downregulated in these EBs than in undifferentiated ESCs, EGFP expression detected in the EBs indicates the presence of PGCs, but not the presence of undifferentiated ESCs. *Rhox6* as well as other PGC markers including *Oct3/4, Rnh2, Piwil2*, and *Fgls* were downregulated in the EBs expressing the shRNA against the *Rhox6* transcripts, whereas the EBs expressing the control shRNA with EGFP-positive cells expressed *Rhox6* and these PGC markers. Collectively, these *in vitro* studies suggest that downregulation of *Rhox6* impairs differentiation of PGCs.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman, 1981; Martin, 1981). ES cells possess two unique characteristics that distinguish them from other organ-specific stem cells identified to date (Smith, 2001; Keller, 2005). First, ESCs are capable of self-renewing while maintaining their undifferentiated state and retaining the normal karyotype. Second, ESCs are pluripotent as they can generate almost all of the cell types in the body (Keller, 2005). Pluripotency of mouse ESCs has been demonstrated by injecting them into a host blastocyst, which resulting in their contribution to all cell types in the adult chimera, including germ cells (Bradley et al., 1984). In addition, ESCs also display a remarkable capability to produce a wide range of well-defined cell types in culture (Smith, 2001). Due to their properties of self-renewal and differentiation, along with the technique to modify their genome (Roshon et al., 2003; Schaniel et al., 2006), ESCs provide an excellent in vitro model for early mammalian development.

The POU domain transcription factor *Oct3/4* is a key regulator of pluripotency, and is expressed in the preimplantation embryo, undifferentiated ESCs and germline cells, but not in

other differentiated somatic cells (Scholer et al., 1990; Yeom et al., 1996; Pesce and Scholer, 2001). Due to its unique expression pattern, both examining expression of *Oct3/4* and using its promoter have been a useful tool to monitor the presence of pluripotent cells as well as differentiation of germ cells in vitro. For example, a genetically modified ES cell line that expresses the enhanced green fluorescent protein (EGFP) under the promoter of *Oct3/4* (Walker et al., 2007), serves as a tool to study differentiation of ESCs as well as germ cells.

It has been demonstrated in previous chapter that ESCs are considered a powerful tool as to study gene function during germ cell differentiation events when combined with shRNA technology. Furthermore, to enrich the differentiation of germ cells from ES cells, ES cells were cultured as aggregates called embryoid bodies (EBs) in the culture medium that induces differentiation of ESCs (West et al., 2006). Since germ cells differentiated in EBs will express EGFP under the promoter of *Oct3/4*, we were able to monitor germ cell differentiation in real-time.

Materials and Methods

Cell culture

The OGR1 embryonic stem cell line is provided by Dr.William L. Stanford at the University of Toronto, ON, Canada. The cell line is routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 15% fetal bovine serum (FBS; Invitrogen), non-essential amino acids (NEAA; Invitrogen), glutamax I (Invitrogen), sodium pyruvate (Invitrogen), penicillin-streptomycin (Sigma), 2-Mercaptoethanol (2-ME, Sigma), and $1x10^7$ units LIF (Chemicon). The cells are changing culture medium everyday and are expanding every other day when they are reaching 80% confluence and are then replating at a seeding density of 1:6.

For Embryoid body (EB) differentiation, ESCs were digested with Trypsin, and then hanging drops are made in the absence of the Leukemia inhibitory factor (LIF) to produce EBs. To enrich germ cell differentiation within EBs, EBs were pooled after 3 days of hanging drops and cultured on an orbital shaker to prevent cells from adhesion. The EB culture medium was supplemented with Ascorbic acid, 1-Thioglycerol, and Transferrin to enrich the differentiation of germ cells within EBs (West et al., 2006). EBs were collected 10 days after cultured on the shaker and their fluorescence was observed under an inverted microscope equipped with an epi-fluorescence lamp (Leica).

Electroporation

The ESCs were digested with Trypsin, then collect the suspension into a tube and bring up a total volume to exactly 10ml. 2~5 x 10^7 cells were taken out and centrifuged at 1000rcf for 5min. Re-suspend the pellet in approximately 0.75ml PBS/2 x 10^7 cells per sample into electroporation cuvette, then add 50ul shRNA vector to bring up a total volume of total 0.8ml. Followed by 10min of incubation on ice. The samples were then electroporated by 12 electrical pulses at a voltage of 325V. After electroporation, the cells were incubated on ice for 10 minutes, and then transfer into gelatin prepared plate with ES medium. Drug selection starts on the following day. After seven days of drug selection, individual puromycin-resistant subclones were collected and cultured in 96-well format. Each clones was used to generate three EBs as described (West et al., 2006).

Construction of shRNA-expression vector

phH1CCP was double digested with KpnI and HindIII (New England Biolabs) enzymes. The digested vector was collected by agarose gel electrophoresis and purified with Agarose Gel DNA Extraction Kit. The purified vector was then proceeded with DNA ligation. Oligonucleotides for shRNA were designed within the full-length transcript of *Rhox6* or *Rhox9*. Oligonucleotides encoding the sequence of senses and antisense strand of target sites were annealed in the presence of 50nM Na^+ at 54-72^{o}C depending on the GC-content of the sequence. T4 ligase (Invotrogen) was used to ligate KpnI/HindIII-digested phH1CCP vector and oligonucleotides were annealed with KpnI/HindIII sites at the ends. Scrambled oligonucleotides were designed from the target sequence and used as control.

Transformation and screening of the constructed shRNA vectors

Competent DH5α cells were thawed on ice and mixed with the products of ligation reaction. The cells were incubated for 42 seconds at 42^{o}C, followed by chilling on ice quickly and sent for shaking at 37^{o}C for 1 hour with SOC medium. The product were then placed unto LB plate with Ampicillin (50ug/ml) and incubated at 37^{o}C overnight. To identify if the clones that have shRNA target sequence correctly ligated into the vector, each Ampicillin-resistant clone was picked up and screened by PCR with Phusion DNA polymerase (Finnzymes). The oligonucleotide used to clone human H1 promoter (RVXhohH1-F) and the ones designed for antisense sequence of each shRNA target were used as primers. The PCR was carried out in the following conditions: initial denaturation at 98^{o}C for 1.5min, 5 cycles of 98^{o}C for 1min, 60^{o}C for 1min and 72^{o}C for 1min, 30 cycles of 98^{o}C for 10sec, 60^{o}C for 30sec and 72^{o}C for 30sec, and final extension at 72^{o}C for 7.5min. PCR were carried out on a thermal cycler (MJ research). PCR

products were analyzed on 1.2% agarose gel in a 96-well format. Clones that show distinct single bands at around 200bp were picked up for purification of the plasmids by the alkaline lysis method. Each shRNA sequence was further validated by PCR with the purified plasmid and with the same set of primers as described above and sequencing. Then the shRNAs were electroporated into undifferentiated OGR1 cells for further study.

Results

The induction of germ cell differentiation in embryoid bodies (EBs).

After 10 days culture of EBs on orbital shaker by applying published method (West et al., 2006), we obtained putative germ cell population within EBs derived from OGR1 cell line. Some of the EBs is observed by strong green fluorescence while some of them by low green fluorescence. The representative examples of EBs are shown in Fig.3.1. Because both germ cells differentiated from OGR1 and undifferentiated OGR1 ESCs in EBs are expressing EGFP, we further validated the differentiation of PGCs in EBs. We extracted RNA from both undifferentiated OGR1 ESCs and EBs and applied semiqRT-PCR with specific marker genes that are expressed in somatic cells in gonad and germ cells (Wang, 2001; Geijsen et al., 2004). Although ESCs express many germ cell-specific genes, true germ cell-specific markers that are not expressed in ESCs are yet to be identified. However, notably, the *Rex-1* (*Zfp-42*) gene, a marker of undifferentiated cells that encodes an acidic zinc finger protein (Hosler et al., 1989; Hosler et al., 1993; Ben-Shushan et al., 1998), is downregulated in EBs compared to its expression in undifferentiated ESCs. In contrast, the *Sry*, a marker for somatic program (Brennan and Capel, 2004), the expression of which is upregulated in EBs (Fig. 3.2). The upregulation of *Sry* and the downregulation of *Rex-1* indicate the differentiated status of cells in EBs and the

remaining undifferentiated ESCs have been minimized at most. Furthermore, along with the expression of other germ cell marker in EBs including *Esg1* (Western et al., 2005; Tanaka et al., 2006), *Fgls, Nanog* (Hatano et al., 2005; Chambers et al., 2007), *Oct3/4* (Kehler et al., 2004; Okamura et al., 2008), *Piwil2* (Lee et al., 2006a; Lee et al., 2006b), *Rnf17, Dazl* (Collier et al., 2005; Nicholas et al., 2009), *Rnh2, Rhox6* and *Rhox9* (Wang et al., 2001; Geijsen et al., 2004), the evidences altogether validated the germ cell differentiation in EBs.

Downregulated expression of *Rhox6* affected stem cell colony formation

Individual subclones consistently expressing downregulated *Rhox6* were isolated after seven days drug selection and were cultured in the 96-well format. Interestingly, we found that the morphology of the colonies differs between the treatment and the controls (Fig. 3.3B). It is shown that nearly 50% of the subclones in shRhox6- and shRhox6/9-OGR1 contained large population of round shaped colonies, whereas only 25% of the subclones in the control group and 9.68% of the subclones in the shRhox9-OGR1 showed similar pattern (Fig. 3.3B). On the other hand, we have found that there were nearly 24% of subclones in the control group and 35% of subclones in shRhox9-OGR1 contained large population of irregular shaped colonies, while in shRhox6- and shRhox6/9-OGR1 there were only 10% subclones showed the similar pattern. Besides, all the four groups showed high frequency of subclones that contained both large numbers of round shaped and irregular shaped colonies. The regular shaped stem cell colonies showed uniformly strong EGFP fluorescence, which indicates the undifferentiated state of ESCs.

Downregulation of *Rhox6* expression prevents the germ cell differentiation in EBs.

To investigate the effect of down-regulation of *Rhox6* expression in the germ cell differentiation from ESCs, our lab built a series of short-hairpin RNA (shRNA) -expression vectors (Siomi and Siomi, 2009). We have built a shRNA-expression vector, phH1CCP (Fig. A2). The vector contains H1 promoter to express the shRNA (Brummelkamp et al., 2002; Kunath et al., 2003), yet it independently expresses puromycin resistant gene (*Puro*^{r}) that is fused with the cyan fluorescent protein (CFP). With the phH1CCP, our lab designed several different shRNA-expression constructs. 1) shRNA against *Rhox6*, named shRhox6-OGR1; 2) shRNA against *Rhox9*, named shRhox9-OGR1; 3) shRNA against *Rhox6* and *Rhox9*, named shRhox6/9-OGR1 hereafter; 4) shRNA against scrambled oligonucleotide sequence as the control, named control –OGR1 hereafter.

To test if the down-regulation of *Rhox6* reduces the number of germ cell differentiated in EBs, those shRNA-expression vectors were electroporated into undifferentiated OGR1 ESCs. Drug selection began in the following day. After seven days puromycin selection, we isolated individual puromycin resistant subclones and cultured them in the 96-well format. EBs were generated from each individual clones (West et al., 2006) and the numbers of fluorescent EBs were then compared among shRhox6, shRhox6/9 and control group.

In sum, 48, 42, 60 measurable EBs in total were generated separately from 31, 27, 34 individual subclones collected from Control, shRhox6 and shRhox6/9-OGR1, respectively (Fig. 3.4). By observing the green fluorescence under the microscope, we found that there were forty one percent EBs derived from control-OGR1 exhibited uniformly strong EGFP expression. However, only about two percent of EBs derived from shRhox6-OGR1 showed strong EGFP expression. In addition, around eight percent of EBs derived from shRhox6/9 displayed

uniformly strong EGFP expression. There were nearly sixty percent EBs derived from shRhox9-OGR1 showed high green fluorescence and there is no significant difference between shRhox9-OGR1 and the control group (Fig. 3.4). Irregular shaped EBs, partial fluorescent EBs and uniformly expressing EGFP EBs with medium level fluorescence were discarded in data collection.

To correlate the EGFP expression with germ cell marker expression in EBs, we pooled EBs of high fluorescent and low fluorescent from control-, shRhox6-, shRhox6/9-OGR1 and extract RNA from them. Then we applied seminq-PCR to investigate the expression of gene markers in EBs (Fig. 3.5). We found that the expression of *Oct3/4* was downregulated and the expressions of other germ cell markers except *Dazl* were suppressed in the EBs with low EGFP expression, whereas, all the markers investigated were properly expressed in the EBs with high EGFP expression derived from control-OGR1 cells. The result validates that the EGFP expression reflects not only *Oct3/4* expression but also germ cell differentiation in EBs. Collectively, the data describe above shows that the downregulation of *Rhox6* reduced the number of germ cells differentiated in EBs.

Discussion

Differentiation of extraembryonic cells may take place in EBs

EB-based differentiation is a powerful system to study in vivo embryonic development as well as the effects of cell-cell interactions and spatial organization on cell-type commitment (Bauwens et al., 2008). EBs recapitulates many aspects of embryonic development (Keller, 1995) for the in vitro lineage potential in generating each of three germ layers (Itskovitz-Eldor et al., 2000). EBs also recapitulates the formation of the proamniotic cavity and this allowed the study

of the mechanism that underlies egg-cylinder formation (Coucouvanis and Martin, 1995). Moreover, recent studies demonstrate that the precursors of germ cells are also induced in EBs (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004).

As we investigated the expression pattern of germ cell markers between undifferentiated ESCs and EBs, interestingly, we found that the expression pattern of them were not identical. The expression of *Esg1, Fgls, Nanog, Oct3/4, Piwil2* and *Rhf12* were either maintained or slightly downregulated in EBs compared to their expression in undifferentiated cells. In contrast, the expression of *Dazl, Rnh2, Rhox6* and *Rhox9* were upregulated in EBs, which indicates that there might be other cell types differentiated other than gonadal somatic cells and germline cells (Fig. 3.2). Notably, the *Rex-1* gene, the marker of undifferentiated ESCs, has been suggested play a role involved in trophoblast development and spermatogenesis for detectable amount of transcript was found in trophoblast and in meiosis germ cells (Rogers et al., 1991). Besides, presumably, the 10 days EBs collected in this study are suggested representing the stage of migratory and post-migratory PGCs due to the imprint erasure analysis (West et al., 2006), although the exact state of germ cells might appear variable because of the undefined factors such as cell diversification, increase in cell density and cellular interaction (Sawada et al., 2006; Nishikawa et al., 2007). Thus, the detectable expression of *Rex1* in EBs (Fig. 3.2) might not only indicate the differentiation of ESCs but also the emergence of trophoblast development within the EBs. The assumption is further validated by the upregulated expression of *Rhox6* in EBs, as *Rhox6* is highly expressed and is restricted in extraembryonic tissues such as the placenta and amnion (Chun et al., 1999; Han, 1998; Tanaka, 2000; MacLean, 2005). Collectively, these data indicate that in addition to the germline lineage, the development of extraembryonic cells might also take place in EBs.

The expression of *Rhox6* does not guarantee the differentiation of germ cells

As 41% of EBs derived from control-OGR1 exhibits uniformly strong EGFP expression, however, there is correspondingly 59% of EBs observed no/low EGFP expression (Fig. 3.4). The 59% of EBs with no EGFP that derived from control-OGR1 exhibit normal expression of *Rhox6,* whereas dramatically repressed expression of other germ cell markers. The result indicates that the expression of *Rhox6* itself does not guarantee the maintaining of *Oct3/4* expression or germ cell differentiation. Furthermore, because *Rhox6* is also highly expressed in extraembryonic cells and cell fates within EBs are hard to control, for example, all cellular interactions — both with the underlying matrix and with other cells — will introduce a range of different physical forces such as tension and shearing forces, which could have profound effects on the differentiation and survival of cells (Sawada et al., 2006), other cell types such as extraembryonic cells might also exist in the EBs, too.

***Rhox6* and *Rhox9* might involved in different pathways**

Interestingly, the expression of *Dazl* was found in the EBs derived from shRhox6/9-OGR1, whereas its expression was suppressed either in the EBs derived from control-OGR1 with no EGFP expression or in the EBs derived from shRhox6-OGR1 (Fig.3.5). The results indicate that the expression of *Dazl* is independent of *Rhox6* expression but somehow associated with *Rhox9* expression and *Oct3/4* expression. *Dazl* proteins are germ-cell-specific RNA-binding proteins essential for gametogenesis and they appear to function in the cytoplasm (Collier et al., 2005). Although *Rhox6* and *Rhox9* are highly homologous genes, the results indicate that they might be involved in different pathways and thus function differently. It may also help to explain why *Rhox9*-deficient mice function normally whereas *Rhox6* mutants exhibit serious reproductive

defects (Daggag et al., 2008). However, the underlying mechanism by which the genes are interacting with each other remained to be determined.

The morphology of the colony might have affected germ cell differentiation

Notably, the morphology of the colony differs between the shRhox6-, shRhox6/9-OGR1 and the control groups. Nearly 50% of subclones in shRhox6- and shRhox6/9-OGR1 contained large population of round shaped colonies, whereas much less percentage of the subclones in the control group and in the shRhox9-OGR1 showed similar pattern. On the contrast, the percentage of subclones containing irregular shaped colonies in the control group was higher than that of shRhox6- and shRhox6/9-OGR1 (Fig. 3.3B). Because *Rhox9*-deficient mice exhibit normal reproduction (Daggag et al., 2008), the shRhox6-OGR1 can be used as an internal control. It has been reported that *Rhox6/9* is heterogeneously expressed in undifferentiated embryonic stem cells culture (Carter et al., 2008), which may explain the increased percentage of "+" subclones and decreased percentage of "-"subclones in shRhox6- and shRhox6/9-OGR1 (Fig. 3.3B). The result indicates that the downregulated expression of *Rhox6* affects the morphology of the stem cell colony. In addition, it has been shown that the control of human embryonic stem cell colony and aggregate size heterogeneity influence differentiation trajectories (Bauwens et al., 2008), it is possible that the morphology of the stem cell colony also exert an effect on differentiation status, which impairs germ cell differentiation synergistically. However, the mechanism of *Rhox6* function in stem cell colony morphology and whether or not the morphology of the colony plays a role in germ cell differentiation remain to be exploited.

Figures

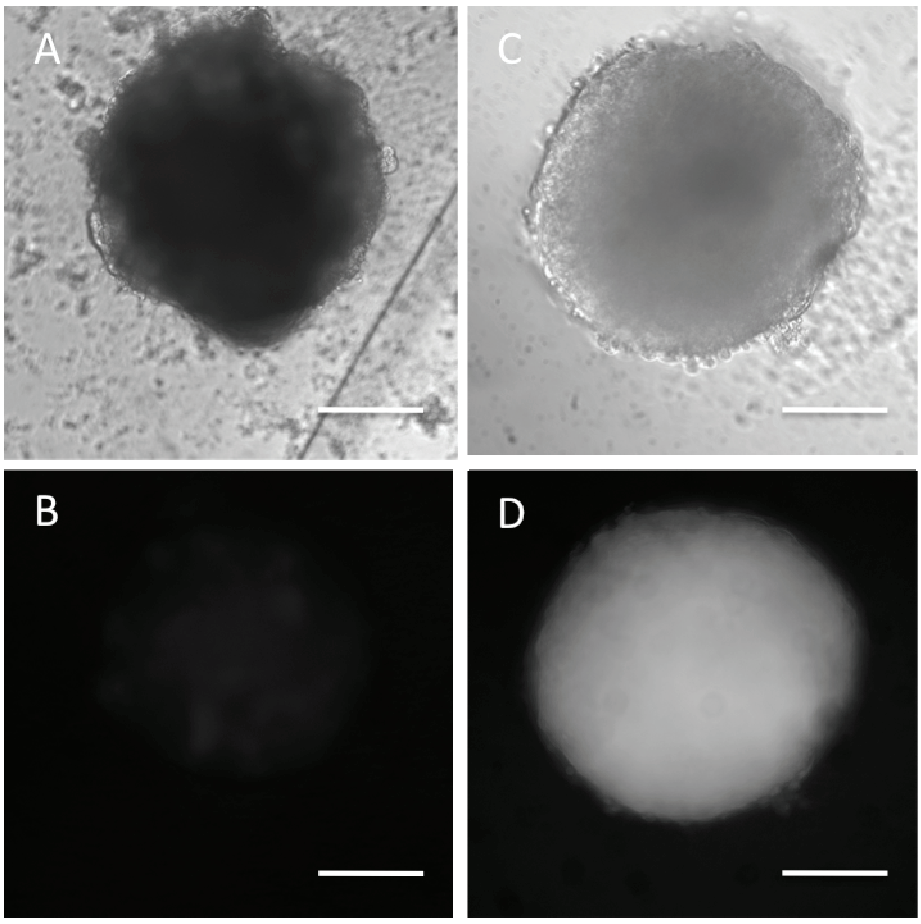


Fig. 3.1. The Embryoid Body (EB) harboring low EGFP expressed cells differentiated within (B) and its phase contract microscope image (A). The EB harboring high EGFP expressed cells within (D) and its phase contract microscope image (C). Scale bar, 200μm.

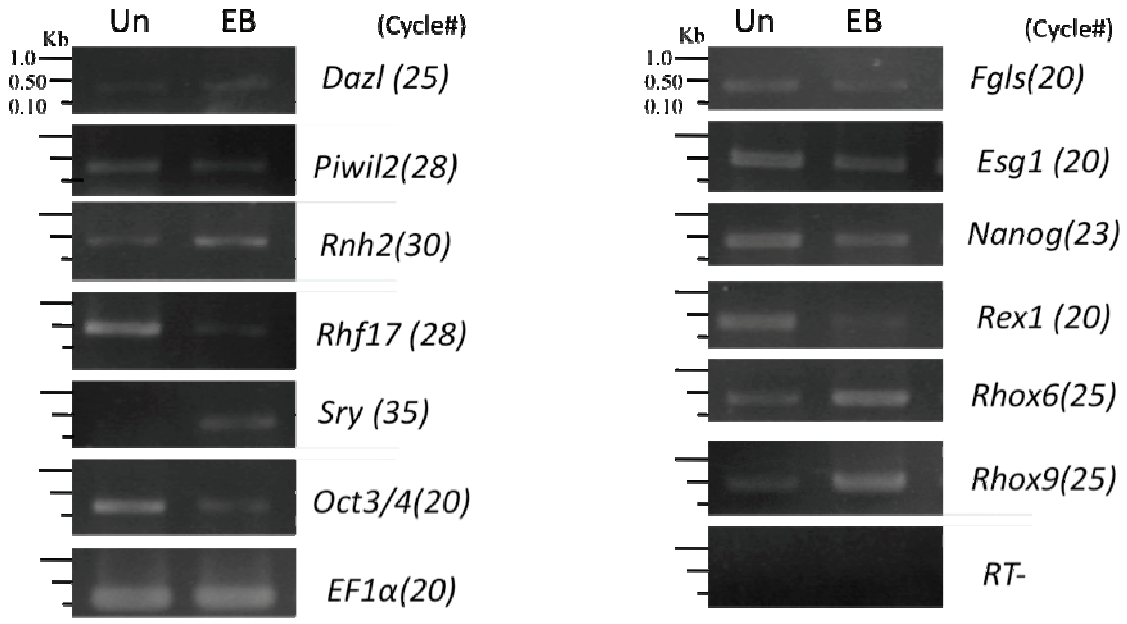
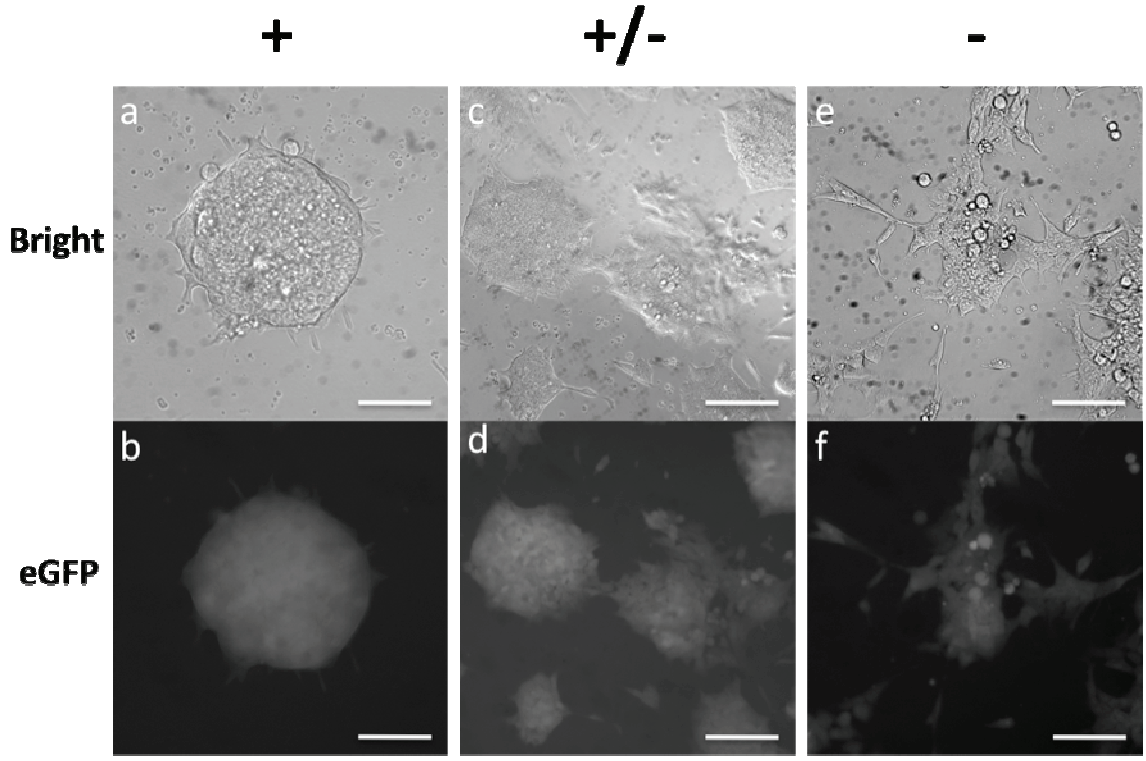


Fig. 3.2. Expression of marker genes for the gonad and germ cells were examined in undifferentiated ESCs and EBs. EF1α is the loading control.

A



B

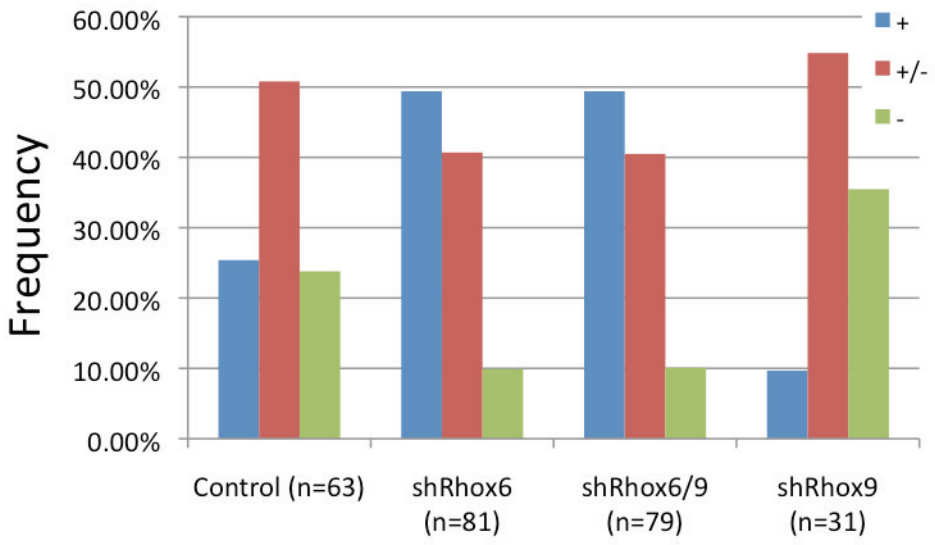


Fig. 3.3. Enhanced green fluorescent protein (EGFP)-expressing ESCs. A, The clones were cataloged as “+”, ”+/-”, ”-”. Clone contains colonies among which over 70% have round shape

(a,b) in the gross was defined as “+”. Clone contains colonies among which over 70% have irregular shape (e,f) on the whole was define as “-”. Clone contains colonies among which irregular shape ones account for 30%-70% in the population was defined as “+/-” (c,d). Colonies merged (<10%) were not considered. Images of cell colonies under Bright light and UV light are shown. Scale bar, 200μm. B, Control, shRhox6,shRhox6/9 and shRhox9 are OGR1 ESCs separately electroporated with scramble DNA, shRNA against *Rhox6*,shRNA against *Rhox6* and *Rhox9* and shRNA against *Rhox9.* shRhox9 serves as an internal control.Percentage of number of clones in different shape is plotted.

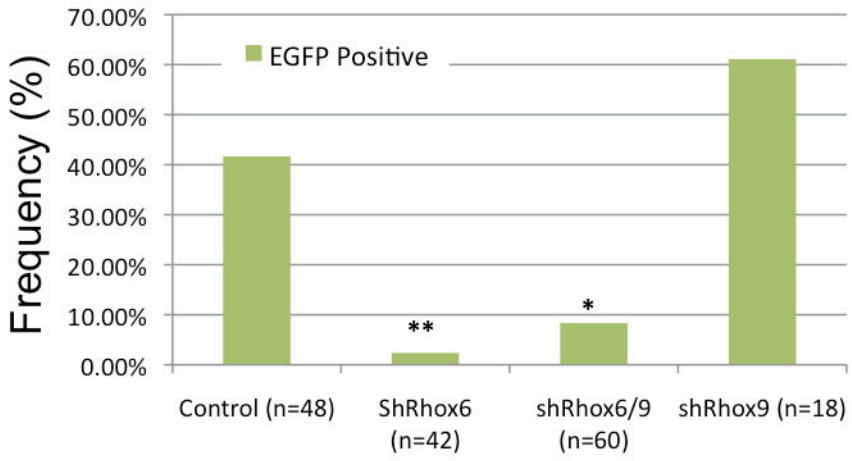


Fig. 3.4. Frequency of EBs with high EGFP expression that derived from Control-, shRhox6-, shRhox6/9 and shRhox9-OGR1. shRhox9-OGR1 serves as an internal control. *: $p<0.001$; **: $p<0.0001$.

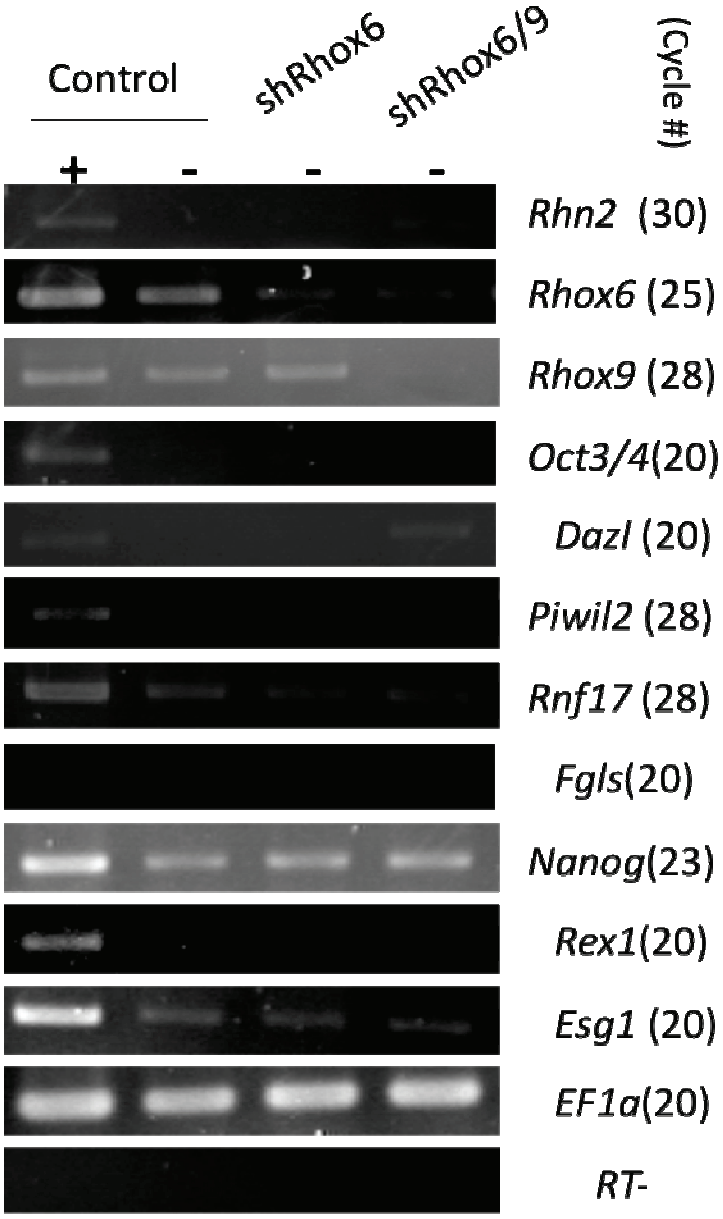


Fig. 3.5. Gene expression pattern in EGFP "High" EBs (+) and in EGFP "Low" EBs (-) derived from Control-, shRhox6-, shRhox6/9-OGR1 were examined. EF1α is the loading control.

CHAPTER 4

Conclusion

The data presented in this thesis are what I have conducted during my graduate studies for Master Degree in the laboratory of Dr. Tetsuya S. Tanaka. The goal of this research was to understand the role of *Rhox6* in the male germ cell differentiation and the research presented in this thesis accomplishes this goal by generating chimeras harboring a hypomorphic mutation in *Rhox6* in vivo and studying downregulated *Rhox6* expression in the differentiation of germ cells from embryonic stem cell in vitro.

The results presented in Chapter 2 described the role of *Rhox6* in maintaining normal phenotype of testes and spermatogenesis in mice. By generating chimeras as the model and through testes and sperm analysis, we found that almost all of the male chimeras exhibited dramatically reduced fertility associated with smaller testes, reduced sperm number and motility and abnormal morphology of the epididymal sperm. In particularly, half of the chimeras were sterile. Those results strongly indicate that the expression of *Rhox6* plays an important role to maintain the normal function of the reproductive organs.

The results in Chapter 3 validated our presumption that expression of *Rhox6* affects germ cell differentiation. By using embryonic stem cells as a model in culture, we successfully derived differentiation of germ cells from ESCs by means of the formation of EBs. The ES cell line (OGR1) used in this research consistently express the enhanced green fluorescent protein (EGFP) under the promoter of *Oct3/4.* By generating EBs from the ESCs that stably express shRNA against *Rhox6* and/or *Rhox9* transcripts, I found that the number of cells that express EGFP in the EBs derived from shRhox6-OGR1 were dramatically reduced compared to the control. With the investigation of marker genes expression pattern between those EBs, we identified that the

expression of EGFP in the cells in EBs indicates the germ cells differentiation within EBs. Therefore, we conclude that the downregulation of *Rhox6* expression prevented the differentiation of germ cells from ESCs.

The research presented in this thesis has contributed to a better understanding of some basic question as to whether or not *Rhox6* plays a role in reproductive organs and if it is, how does the expression of it affects reproduction in mice. The successful use of techniques such as RNA interference and gene trap have made it possible for us to investigate the mechanism of *Rhox6* function both at the cellular level and animal phenotype level.

Despite out current conclusion that the downregulation of *Rhox6* impairs male germ cell differentiation, the results also have raised many other questions that remain to be explored in future studies. One of the problems that needs to be solved is the localization of *Rhox6* expression in embryos and the adult testis of the chimeras and the wild-type. Although localization of *Rhox6* has been described (Han, 1998; Chun et al., 1999; Takasaki, 2000), due to the sequence of *Rhox9* is highly homologous to that of *Rhox6*, it has been difficult to identify the exact localization of *Rhox6* expression. A better understanding of the expression pattern of *Rhox6* will help us to determine whether the expression of *Rhox6* regulates the specification, differentiation or maturation of germ cells.

Furthermore, although we have identified that the expression of *Rhox6* is required for germ cell differentiation, however, whether this expression of *Rhox6* is autonomous or not remains unknown. In addition, because different percentage of chimeras exhibit different degree of defects in phenotype, sperm number and sperm abnormality, those results bring up questions

such as how much *Rhox6* expression is required for germ cell differentiation that remains to be investigated.

A lot of important issues need to be investigated concerning the role of *Rhox6* gene. Since *Rhox6* has been described to have a much more abundant transcript accumulation in female germ cells than in male germ cells in later embryonic development (Takasaki et al., 2000; Daggag et al., 2008), the role that *Rhox6* plays in female gametogenesis remains to be explored. Furthermore, because *Rhox6* is highly expressed in placenta (Tanaka et al., 2000), it will also be of interest to investigate the *Rhox6* function during the development of this tissue. Given the preliminary results we have obtained and the great potential that *Rhox6* holds due to the expression pattern identified to date, the continuing study of *Rhox6* will promisingly provide important insights into the mechanisms at least in regulating male fertility.

APPENDIX A

Figure:

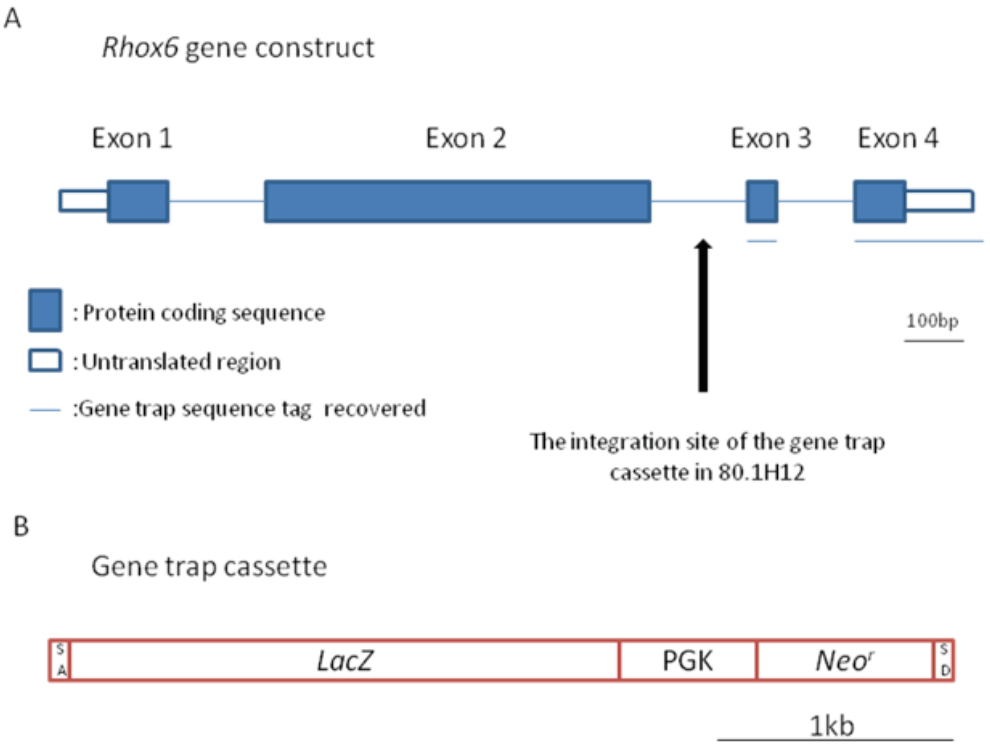


Fig. A1. (A) Schematic representation of the Rhox6 gene structure. (B) The gene trap cassette integrated in 80.1H12. The *Rhox6*gene is about 2.9kb long. Length of introns is not scaled in this figure.

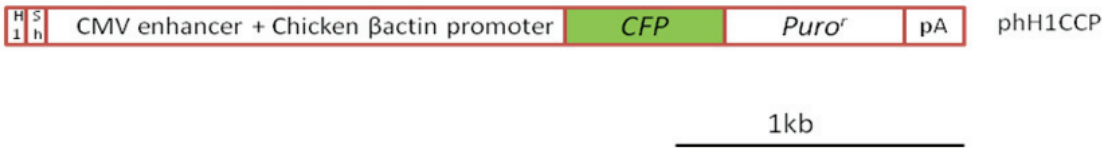


Fig. A2. The vector used in this study.

REFERENCES

Alexandra L. Joyner. (1999). *Gene targeting :A practical approach* (2nd ed. ed.). Oxford ;New York: Oxford University Press, 4-9.

Anderson, R., Copeland, T. K., Scholer, H., Heasman, J., and Wylie, C. (2000). The onset of germ cell migration in the mouse embryo. *Mechanisms of Development, 91*, 61-68.

Aoi, T., Yae, K., Nakagawa, M., Ichisaka, T., Okita, K., Takahashi, K., Chiba, T., Yamanaka, S. (2008). Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science (New York, N.Y.), 321*, 699-702.

Babaie, Y., Herwig, R., Greber, B., Brink, T. C., Wruck, W., Groth, D., Lehrach, H., Burdon, T., Adjaye, J. (2007). Analysis of Oct4-dependent transcriptional networks regulating self-renewal and pluripotency in human embryonic stem cells. *Stem Cells (Dayton, Ohio), 25*, 500-510.

Bauwens, C. L., Peerani, R., Niebruegge, S., Woodhouse, K. A., Kumacheva, E., Husain, M., Zandstra, P. W. (2008). Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories. *Stem Cells (Dayton, Ohio), 26*, 2300-2310.

Ben-Shushan, E., Thompson, J. R., Gudas, L. J., and Bergman, Y. (1998). Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via oct-3/4 and oct-6 binding to an octamer site and a novel protein, rox-1, binding to an adjacent site. *Molecular and Cellular Biology, 18*, 1866-1878.

Besmer, P., Manova, K., Duttlinger, R., Huang, E. J., Packer, A., Gyssler, C., Bachvarova, R. F. (1993). The kit-ligand (steel factor) and its receptor c-kit/W: Pleiotropic roles in gametogenesis and melanogenesis. *Development (Cambridge, England).Supplement,* 125-137.

Bhasin, S., de Kretser, D. M., and Baker, H. W. (1994). Clinical review 64: Pathophysiology and natural history of male infertility. *The Journal of Clinical Endocrinology and Metabolism, 79*, 1525-1529.

Bortvin, A., Eggan, K., Skaletsky, H., Akutsu, H., Berry, D. L., Yanagimachi, R., Page, D. C., Jaenisch, R. (2003). Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. *Development (Cambridge, England), 130*, 1673-1680.

Bradley, A., Evans, M., Kaufman, M. H., and Robertson, E. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature, 309*, 255-256.

Branford, W. W., Zhao, G. Q., Valerius, M. T., Weinstein, M., Birkenmeier, E. H., Rowe, L. B. (1997). Spx1, a novel X-linked homeobox gene expressed during spermatogenesis. *Mechanisms of Development, 65*, 87-98.

Brennan, J., and Capel, B. (2004). One tissue, two fates: Molecular genetic events that underlie testis versus ovary development. *Nature Reviews.Genetics, 5*, 509-521.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science (New York, N.Y.), 296*, 550-553.

Bucay, N., Yebra, M., Cirulli, V., Afrikanova, I., Kaido, T., Hayek, A., Montgomery, A. M. (2009). A novel approach for the derivation of putative primordial germ cells and sertoli cells from human embryonic stem cells. *Stem Cells (Dayton, Ohio), 27*, 68-77.

Carrasco, A. E., McGinnis, W., Gehring, W. J., and De Robertis, E. M. (1984). Cloning of an X. laevis gene expressed during early embryogenesis coding for a peptide region homologous to drosophila homeotic genes. *Cell, 37*, 409-414.

Carter, M. G., Stagg, C. A., Falco, G., Yoshikawa, T., Bassey, U. C., Aiba, K., Sharova, L.V., Shaik, N. (2008). An in situ hybridization-based screen for heterogeneously expressed genes in mouse ES cells. *Gene Expression Patterns : GEP, 8*, 181-198.

Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. *Nature, 450*, 1230-1234.

Chang, H., Brown, C. W., and Matzuk, M. M. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocrine Reviews, 23*, 787-823.

Chang, H., and Matzuk, M. M. (2001). Smad5 is required for mouse primordial germ cell development. *Mechanisms of Development, 104*, 61-67.

Chu, G. C., Dunn, N. R., Anderson, D. C., Oxburgh, L., and Robertson, E. J. (2004). Differential requirements for Smad4 in TGFbeta-dependent patterning of the early mouse embryo. *Development (Cambridge, England), 131*, 3501-3512.

Chun, J. Y., Han, Y. J., and Ahn, K. Y. (1999). Psx homeobox gene is X-linked and specifically expressed in trophoblast cells of mouse placenta. *Developmental Dynamics : An Official Publication of the American Association of Anatomists, 216*, 257-266.

Clark, A. M., Garland, K. K., and Russell, L. D. (2000). Desert hedgehog (dhh) gene is required in the mouse testis for formation of adult-type leydig cells and normal development of peritubular cells and seminiferous tubules. *Biology of Reproduction, 63*, 1825-1838.

Collier, B., Gorgoni, B., Loveridge, C., Cooke, H. J., and Gray, N. K. (2005). The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. *The EMBO Journal, 24*, 2656-2666.

Cooke, H. J., and Saunders, P. T. (2002). Mouse models of male infertility. *Nature Reviews.Genetics, 3*, 790-801.

Coucouvanis, E., and Martin, G. R. (1995). Signals for death and survival: A two-step mechanism for cavitation in the vertebrate embryo. *Cell, 83*, 279-287.

Covello, K. L., Kehler, J., Yu, H., Gordan, J. D., Arsham, A. M., Hu, C. J., Labosky, P. A., Simon, M. C., Keith, B. (2006). HIF-2alpha regulates oct-4: Effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes and Development, 20*, 557-570.

Cullen, B. R. (2005). RNAi the natural way. *Nature Genetics, 37*, 1163-1165.

Dadoune, J. P., Siffroi, J. P., and Alfonsi, M. F. (1994). Ultrastructural localization of rDNA and rRNA by in situ hybridization in the nucleolus of human spermatids. *Cell and Tissue Research, 278*, 611-616.

Daggag, H., Svingen, T., Western, P. S., van den Bergen, J. A., McClive, P. J., Harley, V. R., Koopman, P., Sinclair, A. H. (2008). The rhox homeobox gene family shows sexually dimorphic and dynamic expression during mouse embryonic gonad development. *Biology of Reproduction, 79*, 468-474.

Dann, C. T., Alvarado, A. L., Molyneux, L. A., Denard, B. S., Garbers, D. L., and Porteus, M. H. (2008). Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. *Stem Cells (Dayton, Ohio), 26*, 2928-2937.

De Miguel, M. P., Cheng, L., Holland, E. C., Federspiel, M. J., and Donovan, P. J. (2002). Dissection of the c-kit signaling pathway in mouse primordial germ cells by retroviral-mediated gene transfer. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 10458-10463.

Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W., and Kemler, R. (1985). The in vitro development of blastocyst-derived embryonic stem cell lines: Formation of visceral yolk sac, blood islands and myocardium. *Journal of Embryology and Experimental Morphology, 87*, 27-45.

Donovan, P. J. (1998). The germ cell--the mother of all stem cells. *The International Journal of Developmental Biology, 42*, 1043-1051.

Durcova-Hills, G., Adams, I. R., Barton, S. C., Surani, M. A., and McLaren, A. (2006). The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. *Stem Cells (Dayton, Ohio), 24*, 1441-1449.

Durcova-Hills, G., Tang, F., Doody, G., Tooze, R., and Surani, M. A. (2008). Reprogramming primordial germ cells into pluripotent stem cells. *PloS One, 3*, 3531-3541.

Evans, M. J., and Kaufman, M. H. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature, 292*, 154-156.

Extavour, C. G., and Akam, M. (2003). Mechanisms of germ cell specification across the metazoans: Epigenesis and preformation. *Development (Cambridge, England), 130*, 5869-5884.

Ferlin, A., Raicu, F., Gatta, V., Zuccarello, D., Palka, G., and Foresta, C. (2007). Male infertility: Role of genetic background. *Reproductive Biomedicine Online, 14*, 734-745.

Fujimoto, T., Miyayama, Y., and Fuyuta, M. (1977). The origin, migration and fine morphology of human primordial germ cells. *The Anatomical Record, 188*, 315-330.

Fujiwara, T., Dunn, N. R., and Hogan, B. L. (2001). Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. *Proceedings of the National Academy of Sciences of the United States of America, 98*, 13739-13744.

Gehring, W. J., Affolter, M., and Burglin, T. (1994). Homeodomain proteins. *Annual Review of Biochemistry, 63*, 487-526.

Gehring, W. J., and Hiromi, Y. (1986). Homeotic genes and the homeobox. *Annual Review of Genetics, 20*, 147-173.

Geijsen, N., Horoschak, M., Kim, K., Gribnau, J., Eggan, K., and Daley, G. Q. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature, 427*, 148-154.

Giadrossi, S., Dvorkina, M., and Fisher, A. G. (2007). Chromatin organization and differentiation in embryonic stem cell models. *Current Opinion in Genetics and Development, 17*, 132-138.

Gilbert, S. F. (2000). *Developmental biology* (Sixth ed. ed.). Sunderland: Sinauer Associates, Inc.,124-126.

Godin, I., Deed, R., Cooke, J., Zsebo, K., Dexter, M., and Wylie, C. C. (1991). Effects of the steel gene product on mouse primordial germ cells in culture. *Nature, 352*, 807-809.

Gomperts, M., Garcia-Castro, M., Wylie, C., and Heasman, J. (1994). Interactions between primordial germ cells play a role in their migration in mouse embryos. *Development (Cambridge, England), 120*, 135-141.

Grimaldi, P., Rossi, P., Dolci, S., Ripamonti, C. B., and Geremia, R. (2002). Molecular genetics of male infertility: Stem cell factor/c-kit system. *American Journal of Reproductive Immunology (New York, N.Y.: 1989), 48*, 27-33.

Guan, K., Nayernia, K., Maier, L. S., Wagner, S., Dressel, R., Lee, J. H., Nolte, J., Wolf, F., Li, M., Engel, W., Hasenfuss, G. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature, 440*, 1199-1203.

Han, Y. J., Park, A. R., Sung, D. Y., and Chun, J. Y. (1998). Psx, a novel murine homeobox gene expressed in placenta. *Gene, 207*, 159-166.

Han, Y. J., Lee, Y. H., and Chun, J. Y. (2000). Identification and characterization of psx-2, a novel member of the psx (placenta-specific homeobox) family. *Gene, 241*, 149-155.

Hatano, S. Y., Tada, M., Kimura, H., Yamaguchi, S., Kono, T., Nakano, T., Suemori, H., Nakatsuji, N., Tada, T. (2005). Pluripotential competence of cells associated with nanog activity. *Mechanisms of Development, 122*, 67-79.

Hayashi, K., Kobayashi, T., Umino, T., Goitsuka, R., Matsui, Y., and Kitamura, D. (2002). SMAD1 signaling is critical for initial commitment of germ cell lineage from mouse epiblast. *Mechanisms of Development, 118*, 99-109.

Hayashi, Y., Hayashi, M., and Kobayashi, S. (2004). Nanos suppresses somatic cell fate in drosophila germ line. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 10338-10342.

Hogan, B. (1994). *Manipulating the mouse embryo :A laboratory manual* (2nd ed. ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 314-345.

Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E., and Prydz, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Research, 30*, 1757-1766.

Hosler, B. A., LaRosa, G. J., Grippo, J. F., and Gudas, L. J. (1989). Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Molecular and Cellular Biology, 9*, 5623-5629.

Hosler, B. A., Rogers, M. B., Kozak, C. A., and Gudas, L. J. (1993). An octamer motif contributes to the expression of the retinoic acid-regulated zinc finger gene rex-1 (zfp-42) in F9 teratocarcinoma cells. *Molecular and Cellular Biology, 13*, 2919-2928.

Hu, Z., MacLean, J. A., Bhardwaj, A., and Wilkinson, M. F. (2007). Regulation and function of the Rhox5 homeobox gene. *Annals of the New York Academy of Sciences, 1120*, 72-83.

Hubner, K., Fuhrmann, G., Christenson, L. K., Kehler, J., Reinbold, R., De La Fuente, R., Boiani, M. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science (New York, N.Y.), 300*, 1251-1256.

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreg, H., Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Molecular Medicine (Cambridge, Mass.), 6*, 88-95.

Kawai, Y., Hata, T., Suzuki, O., and Matsuda, J. (2006). The relationship between sperm morphology and in vitro fertilization ability in mice. *The Journal of Reproduction and Development, 52*, 561-568.

Kehler, J., Tolkunova, E., Koschorz, B., Pesce, M., Gentile, L., Boiani, M., Lomeli, H., Nagy, A., McLaughlin, K. J., Scholer, H. R., Tomilin, A. (2004). Oct4 is required for primordial germ cell survival. *EMBO Reports, 5*, 1078-1083.

Keith, B., and Simon, M. C. (2007). Hypoxia-inducible factors, stem cells, and cancer. *Cell, 129*, 465-472.

Keller, G. (2005). Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. *Genes Development, 19*, 1129-1155.

Keller, G. M. (1995). In vitro differentiation of embryonic stem cells. *Current Opinion in Cell Biology, 7*, 862-869.

Kelly, S. J. (1977). Studies of the developmental potential of 4- and 8-cell stage mouse blastomeres. *The Journal of Experimental Zoology, 200*, 365-376.

Kmita, M., and Duboule, D. (2003). Organizing axes in time and space; 25 years of colinear tinkering. *Science (New York, N.Y.), 301*, 331-333.

Kobayashi, S., Yamada, M., Asaoka, M., and Kitamura, T. (1996). Essential role of the posterior morphogen nanos for germline development in drosophila. *Nature, 380*, 708-711.

Krzanowska, H., Wabik-Sliz, B., and Rafinski, J. (1991). Phenotype and fertilizing capacity of spermatozoa of chimaeric mice produced from two strains that differ in sperm quality. *Journal of Reproduction and Fertility, 91*, 667-676.

Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T., and Rossant, J. (2003). Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nature Biotechnology, 21*, 559-561.

Larson, J. L., and Miller, D. J. (1999). Simple histochemical stain for acrosomes on sperm from several species. *Molecular Reproduction and Development, 52*, 445-449.

Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P., Hogan, B. L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes and Development, 13*, 424-436.

Lawson, K. A., and Hage, W. J. (1994). Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Foundation Symposium, 182*, 68-84; discussion 84-91.

Lee, J. H., Engel, W., and Nayernia, K. (2006). Stem cell protein Piwil2 modulates expression of murine spermatogonial stem cell expressed genes. *Molecular Reproduction and Development, 73*, 173-179.

Lee, J. H., Schutte, D., Wulf, G., Fuzesi, L., Radzun, H. J., Schweyer, S., Engel, W., Nayernia, K. (2006). Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway. *Human Molecular Genetics, 15*, 201-211.

Levine, M., Rubin, G. M., and Tjian, R. (1984). Human DNA sequences homologous to a protein coding region conserved between homeotic genes of drosophila. *Cell, 38*, 667-673.

Wolpert, L., Beddington, R., Brockes, J., and Jessell, T. (2006). *Principles of development* (3rd ed.) Oxford University Press, 663-684.

Li, Y., Lemaire, P., and Behringer, R. R. (1997). Esx1, a novel X chromosome-linked homeobox gene expressed in mouse extraembryonic tissues and male germ cells. *Developmental Biology, 188*, 85-95.

McLaren, A. (2003). Primordial germ cells in the mouse. *Developmental Biology, 262*, 1-15.

Maclean, J. A.,2nd, Chen, M. A., Wayne, C. M., Bruce, S. R., Rao, M., Meistrich, M. L., Macleod, C., Wilkinson, M. F. (2005). Rhox: A new homeobox gene cluster. *Cell, 120*, 369-382.

Maclean, J. A.,2nd, and Wilkinson, M. F. (2005). Gene regulation in spermatogenesis. *Current Topics in Developmental Biology, 71*, 131-197.

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proceedings of the National Academy of Sciences of the United States of America, 78*, 7634-7638.

Masui, S. (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nature Cell Biology, 9*, 625-632.

McGinnis, W., and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell, 68*, 283-302.

McLaren, A. (1999). Signaling for germ cells. *Genes and Development, 13*, 373-376.

Meistrich, M. L., Kasai, K., Olds-Clarke, P., MacGregor, G. R., Berkowitz, A. D., and Tung, K. S. (1994). Deficiency in fertilization by morphologically abnormal sperm produced by azh mutant mice. *Molecular Reproduction and Development, 37*, 69-77.

Mendoza-Lujambio, I., Burfeind, P., Dixkens, C., Meinhardt, A., Hoyer-Fender, S., Engel, W. (2002). The Hook1 gene is non-functional in the abnormal spermatozoon head shape (azh) mutant mouse. *Human Molecular Genetics, 11*, 1647-1658.

Molyneaux, K. A., Stallock, J., Schaible, K., and Wylie, C. (2001). Time-lapse analysis of living mouse germ cell migration. *Developmental Biology, 240*, 488-498.

Molyneaux, K. A., Zinszner, H., Kunwar, P. S., Schaible, K., Stebler, J., Sunshine, M. J., Raz, E., Littman, D., Wylie, C., Lehmann, R. (2003). The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development (Cambridge, England), 130*, 4279-4286.

Moore, H, and Aflatoonian, B. (2006). Germ cells from mouse and human embryonic stem cells. *Journal of Reproduction and Fertility, 132*, 699.

Nakano, T., Kodama, H., and Honjo, T. (1994). Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science (New York, N.Y.), 265*, 1098-1101.

Nicholas, C. R., Xu, E. Y., Banani, S. F., Hammer, R. E., Hamra, F. K., and Reijo Pera, R. A. (2009). Characterization of a dazl-GFP germ cell-specific reporter. *Genesis (New York, N.Y.: 2000), 47*, 74-84.

Nishikawa, S., Jakt, L. M., and Era, T. (2007). Embryonic stem-cell culture as a tool for developmental cell biology. *Nature Reviews.Molecular Cell Biology, 8*, 502-507.

Nishikawa, S. I., Nishikawa, S., Hirashima, M., Matsuyoshi, N., and Kodama, H. (1998). Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells

at a diverging point of endothelial and hemopoietic lineages. *Development (Cambridge, England), 125*, 1747-1757.

Nocka, K., Majumder, S., Chabot, B., Ray, P., Cervone, M., Bernstein, A., Besmer, P. (1989). Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice--evidence for an impaired c-kit kinase in mutant mice. *Genes and Development, 3*, 816-826.

Noguchi, T., and Noguchi, M. (1985). A recessive mutation (ter) causing germ cell deficiency and a high incidence of congenital testicular teratomas in 129/Sv-ter mice. *Journal of the National Cancer Institute, 75*, 385-392.

Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanvch, T., Nussenzweig, M., Tarakhovsky, A., Saitou, M., Surani, M. A. (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature, 436*, 207-213.

Okamura, D., Tokitake, Y., Niwa, H., and Matsui, Y. (2008). Requirement of Oct3/4 function for germ cell specification. *Developmental Biology, 317*, 576-584.

Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature, 448*, 313-317.

Pesce, M., and Scholer, H. R. (2001). Oct-4: Gatekeeper in the beginnings of mammalian development. *Stem Cells (Dayton, Ohio), 19*, 271-278.

Pitman, J. L., Lin, T. P., Kleeman, J. E., Erickson, G. F., and MacLeod, C. L. (1998). Normal reproductive and macrophage function in pem homeobox gene-deficient mice. *Developmental Biology, 202*, 196-214.

Rogers, M. B., Hosler, B. A., and Gudas, L. J. (1991). Specific expression of a retinoic acid-regulated, zinc-finger gene, rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development (Cambridge, England), 113*, 815-824.

Roshon, M., DeGregori, J. V., and Ruley, H. E. (2003). Gene trap mutagenesis of hnRNP A2/B1: A cryptic 3' splice site in the neomycin resistance gene allows continued expression of the disrupted cellular gene. *BMC Genomics, 4*, 2-11.

Ruberti, I., Sessa, G., Lucchetti, S., and Morelli, G. (1991). A novel class of plant proteins containing a homeodomain with a closely linked leucine zipper motif. *The EMBO Journal, 10*, 1787-1791.

Runyan, C., Shoemaker, A., Surani, A., Wylie, C., and Gu, Y. (2009). Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. *Development, 136*, 1295-1303.

Rutz, S., and Scheffold, A. (2004). Towards in vivo application of RNA interference - new toys, old problems. *Arthritis Research and Therapy, 6*, 78-85.

Saitou, M., Barton, S. C., and Surani, M. A. (2002). A molecular programme for the specification of germ cell fate in mice. *Nature, 418*, 293-300.

Sakurai, T., Iguchi, T., Moriwaki, K., and Noguchi, M. (1995). The ter mutation first causes primordial germ cell deficiency in ter/ter mouse embryos at 8 days of gestation. *Development, Growth Differentiation, 37*, 293-307.

Sato, M., Kimura, T., Kurokawa, K., Fujita, Y., Abe, K., Masuhara, M., Ryo, A., Yamamoto, M., Nakano, T. (2002). Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. *Mechanisms of Development, 113*, 91-94.

Sawada, Y., Tamada, M., Dubin-Thaler, B. J., Cherniavskaya, O., Sakai, R., Tanaka, S., Sheetz, M. P. (2006). Force sensing by mechanical extension of the src family kinase substrate p130Cas. *Cell, 127*, 1015-1026.

Schaniel, C., Li, F., Schafer, X. L., Moore, T., Lemischka, I. R., and Paddison, P. J. (2006). Delivery of short hairpin RNAs--triggers of gene silencing--into mouse embryonic stem cells. *Nature Methods, 3*, 397-400.

Scholer, H. R., Dressler, G. R., Balling, R., Rohdewohld, H., and Gruss, P. (1990). Oct-4: A germline-specific transcription factor mapping to the mouse t-complex. *The EMBO Journal, 9*, 2185-2195.

Schulz, B., Banuett, F., Dahl, M., Schlesinger, R., Schafer, W., Martin, T., Herskowitz, I., Kahmann, R. (1990). The b alleles of U. maydis, whose combinations program pathogenic development, code for polypeptides containing a homeodomain-related motif. *Cell, 60*, 295-306.

Semenza, G. L. (2000). HIF-1 and human disease: One highly involved factor. *Genes and Development, 14*, 1983-1991.

Seydoux, G., and Braun, R. E. (2006). Pathway to totipotency: Lessons from germ cells. *Cell, 127*, 891-904.

Shashikant, C. S., Utset, M. F., Violette, S. M., Wise, T. L., Einat, P., Einat, M., Pendleton, J. W., Schughart, K., Ruddle, F. H. (1991). Homeobox genes in mouse development. *Critical Reviews in Eukaryotic Gene Expression, 1*, 207-245.

Shepherd, J. C., McGinnis, W., Carrasco, A. E., De Robertis, E. M., and Gehring, W. J. (1984). Fly and frog homoeo domains show homologies with yeast mating type regulatory proteins. *Nature, 310*, 70-71.

Siomi, H., and Siomi, M. C. (2009). On the road to reading the RNA-interference code. *Nature, 457*, 396-404.

Smith, A. G. (2001). Embryo-derived stem cells: Of mice and men. *Annual Review of Cell and Developmental Biology, 17*, 435-462.

Stevens, L. C. (1967). Origin of testicular teratomas from primordial germ cells in mice. *Journal of the National Cancer Institute, 38*, 549-552.

Stevens, L. C. (1973). A new inbred subline of mice (129-terSv) with a high incidence of spontaneous congenital testicular teratomas. *Journal of the National Cancer Institute, 50*, 235-242.

Subramaniam, K., and Seydoux, G. (1999). Nos-1 and nos-2, two genes related to drosophila nanos, regulate primordial germ cell development and survival in caenorhabditis elegans. *Development (Cambridge, England), 126*, 4861-4871.

Sutton, K. A., and Wilkinson, M. F. (1997). The rapidly evolving pem homeobox gene and Agtr2, Ant2, and Lamp2 are closely linked in the proximal region of the mouse X chromosome. *Genomics, 45*, 447-450.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell, 131*, 861-872.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*, 663-676.

Takasaki, N., McIsaac, R., and Dean, J. (2000). Gpbox (Psx2), a homeobox gene preferentially expressed in female germ cells at the onset of sexual dimorphism in mice. *Developmental Biology, 223*, 181-193.

Takasaki, N., Rankin, T., and Dean, J. (2001). Normal gonadal development in mice lacking GPBOX, a homeobox protein expressed in germ cells at the onset of sexual dimorphism. *Molecular and Cellular Biology, 21*, 8197-8202.

Tam, P. P., and Snow, M. H. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *Journal of Embryology and Experimental Morphology, 64*, 133-147.

Tam, P. P., and Zhou, S. X. (1996). The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Developmental Biology, 178*, 124-132.

Tanaka, T. S., Jaradat, S. A., Lim, M. K., Kargul, G. J., Wang, X., Grahovac, M. J., Pantano, S., Sano, Y., Piao, Y., Nagaraja, R., Doi, H., Ko, M. S. (2000). Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proceedings of the National Academy of Sciences of the United States of America, 97*, 9127-9132.

Tanaka, S. S., Yamaguchi, Y. L., Tsoi, B., Lickert, H., and Tam, P. P. (2005). IFITM/Mil/fragilis family proteins IFITM1 and IFITM3 play distinct roles in mouse primordial germ cell homing and repulsion. *Developmental Cell, 9*, 745-756.

Tanaka, T. S., Lopez de Silanes, I., Sharova, L. V., Akutsu, H., Yoshikawa, T., Amano, H., Yamanaka, S., Gorospe, M., Ko, M. S. (2006). Esg1, expressed exclusively in preimplantation embryos, germline, and embryonic stem cells, is a putative RNA-binding protein with broad RNA targets. *Development, Growth and Differentiation, 48*, 381-390.

Ten Berge, D., Koole, W., Fuerer, C., Fish, M., Eroglu, E., and Nusse, R. (2008). Wnt signaling mediates self-organization and axis formation in embryoid bodies. *Cell Stem Cell, 3*, 508-520.

Toyooka, Y., Tsunekawa, N., Akasu, R., and Noce, T. (2003). Embryonic stem cells can form germ cells in vitro. *Proceedings of the National Academy of Sciences of the United States of America, 100*, 11457-11462.

Tremblay, K. D., Dunn, N. R., and Robertson, E. J. (2001). Mouse embryos lacking Smad1 signals display defects in extra-embryonic tissues and germ cell formation. *Development (Cambridge, England), 128*, 3609-3621.

Tsuda, M., Sasaoka, Y., Kiso, M., Abe, K., Haraguchi, S., Kobayashi, S., Saga, Y. (2003). Conserved role of nanos proteins in germ cell development. *Science (New York, N.Y.), 301*, 1239-1241.

Turner, C. A.,Jr, Mack, D. H., and Davis, M. M. (1994). Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell, 77*, 297-306.

Vollbrecht, E., Veit, B., Sinha, N., and Hake, S. (1991). The developmental gene knotted-1 is a member of a maize homeobox gene family. *Nature, 350*, 241-243.

Walker, E., Ohishi, M., Davey, R. E., Zhang, W., Cassar, P. A., Tanaka, T. S., Morris, Q., Hughes, T. R., Zandstra, P. W., Stanford, W. L. (2007). Prediction and testing of novel transcriptional networks regulating embryonic stem cell self-renewal and commitment. *Cell Stem Cell, 1*, 71-86.

Wang, P. J., McCarrey, J. R., Yang, F., and Page, D. C. (2001). An abundance of X-linked genes expressed in spermatogonia. *Nature Genetics, 27*, 422-426.

Wang, C., and Lehmann, R. (1991). Nanos is the localized posterior determinant in drosophila. *Cell, 66*, 637-647.

Wang, P. J., McCarrey, J. R., Yang, F., and Page, D. C. (2001). An abundance of X-linked genes expressed in spermatogonia. *Nature Genetics, 27*, 422-426.

Weatherbee, S. D., Halder, G., Kim, J., Hudson, A., and Carroll, S. (1998). Ultrabithorax regulates genes at several levels of the wing-patterning hierarchy to shape the development of the drosophila haltere. *Genes and Development, 12*, 1474-1482.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E., Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature, 448*, 318-324.

West, J. A., Park, I. H., Daley, G. Q., and Geijsen, N. (2006). In vitro generation of germ cells from murine embryonic stem cells. *Nature Protocols, 1*, 2026-2036.

Western, P., Maldonado-Saldivia, J., van den Bergen, J., Hajkova, P., Saitou, M., Barton, S., Surani, M. A. (2005). Analysis of Esg1 expression in pluripotent cells and the germline reveals similarities with Oct4 and Sox2 and differences between human pluripotent cell lines. *Stem Cells (Dayton, Ohio), 23*, 1436-1442.

Wolpert, L. (1991). *The triumph of the embryo*. Oxford [England] ;New York : Oxford University Press, 234-261

Wylie, C. (2000). Germ cells. *Current Opinion in Genetics and Development, 10*, 410-413.

Yabuta, Y., Kurimoto, K., Ohinata, Y., Seki, Y., and Saitou, M. (2006). Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. *Biology of Reproduction, 75*, 705-716.

Yeom, Y. I., Fuhrmann, G., Ovitt, C. E., Brehm, A., Ohbo, K., Gross, M., Scholer, H. R. (1996). Germline regulatory element of oct-4 specific for the totipotent cycle of embryonal cells. *Development (Cambridge, England), 122*, 881-894.

Yeung, C. H., Sonnenberg-Riethmacher, E., and Cooper, T. G. (1999). Infertile spermatozoa of c-ros tyrosine kinase receptor knockout mice show flagellar angulation and maturational defects in cell volume regulatory mechanisms. *Biology of Reproduction, 61*, 1062-1069.

Ying, Y., Liu, X. M., Marble, A., Lawson, K. A., and Zhao, G. Q. (2000). Requirement of Bmp8b for the generation of primordial germ cells in the mouse. *Molecular Endocrinology (Baltimore, Md.), 14*, 1053-1063.

Ying, Y., and Zhao, G. Q. (2001). Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. *Developmental Biology, 232*, 484-492.

Yoshimizu, T., Obinata, M., and Matsui, Y. (2001). Stage-specific tissue and cell interactions play key roles in mouse germ cell specification. *Development (Cambridge, England), 128*, 481-490.

Youngren, K. K., Coveney, D., Peng, X., Bhattacharya, C., Schmidt, L. S., Nickerson, M. L., Lamb, B. T., Deng, J. M., Behringer, R. R., Capel, B., Rubin, E. M., Nadeau, J. H., Matin, A. (2005). The ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. *Nature, 435*, 360-364.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Jonsdottir, G. A., Ruotti, V., Stewart, R., Thomson, J. A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science (New York, N.Y.), 318*, 1917-1920.

Zhang, H. J., Siu, M. K., Wong, E. S., Wong, K. Y., Li, A. S., Chan, K. Y., Ngan, H. Y., Cheung, A. N. (2008). Oct4 is epigenetically regulated by methylation in normal placenta and gestational trophoblastic disease. *Placenta, 29*, 549-554.

Zhao, G. Q. (2003). Consequences of knocking out BMP signaling in the mouse. *Genesis (New York, N.Y.: 2000), 35*, 43-56.

Zheng, H., Stratton, C. J., Morozumi, K., Jin, J., Yanagimachi, R., and Yan, W. (2007). Lack of Spem1 causes aberrant cytoplasm removal, sperm deformation, and male infertility. *Proceedings of the National Academy of Sciences of the United States of America, 104*, 6852-6857.

Zwaka, T. P., and Thomson, J. A. (2005). A germ cell origin of embryonic stem cells? *Development (Cambridge, England), 132*, 227-233.